DOCKET NO: 286808US0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF	:
FRANK MATTNER, ET AL.	EXAMINER: KOLKER
SERIAL NO: 10/571,469	:
FILED: MARCH 13, 2006	ART UNIT: 1649
FOR: APHERESIS DEVICE	:

DECLARATION UNDER 37 CFR 1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

I, Eliezer Masliah, M.D., state:

1. I have substantial experience and expertise in the molecular mechanisms of synaptic pathology in neurodegenerative diseases, especially Alzheimer's Disease (AD), as well as in the development of new experimental therapies for AD. I have been working on this topics concerning AD for more than 20years. (Please see the attached *Curriculum Vitae and Biographical Sketch*).

2. I have conducted research collaboration with the assignees of the above-identified application and I do not have any interest in the outcome of this application. I was not, or am currently not, or will not be compensated monetarily for my time to provide this Declaration.

3. I am familiar with the application and current claims that are pending in the above-identified application in that the claims define a method for treating Alzheimer's Disease (AD) in a patient by contacting blood or plasma flow of a patient suffering from AD

or a patient with a risk for AD with an apheresis device, the apheresis device including a solid carrier, said solid carrier having anti-amyloid-β precursor protein (APP) antibodies attached to a surface of the solid carrier so as to reduce APP polypeptides in the brain and treat AD in the patient.

4. I also understand that the United States Patent and Trademark Office (USPTO) has rejected this method as something that would have been obvious to do to a person in this field based on the publications of DeMattos (2001) (*Proc Natl Acad Sci, USA* 98:8850-8855) and Kojima (2001) (*J Biochem Biophys Methods* 49:241-251).

5. I do not agree with the conclusions of the USPTO and one in this field would not have thought to do what is defined in the claims of this application based on the publications of DeMattos and Kojima.

6. The USPTO misinterpreted some of the data and results of the DeMattos paper and did not take into consideration the difference between an antibody which is administered to a patient and acts intra-corporally and an antibody which acts via immobilization on the solid surface extra-corporally as in the claims of this application.

7. First, the USPTO refers to a "peripheral" administration of m266 and from this I can only infer that the USPTO views that m266 antibody exclusively acts from the periphery (the blood) and does not even occur in the brain or in the CSF. However, this is not true. Even in the DeMattos et al, it is clear that m266 also crosses the blood brain barrier (BBB); the separation of circulating blood and cerebrospinal fluid (CSF) in the central nervous system (CNS); occurring along all capillaries and consisting of tight junctions around the capillaries that do not exist in normal circulation. DeMattos stated on page 8852, right column, that the concentration of the antibody in CSF was 12.0 ± 0.95 ng/ml. Despite the fact that in DeMattos it was speculated that this amount "should only be able to account for an increase of Aβ of less than 1 ng/ml", the DeMattos statement turned out to be rather due to a

detectability problem because in a follow-up publication (see enclosed article of Dodart et al. (Nature Neuroscience 5 (5) (2002): 452- 457) the same group found that the presence of the m266 antibody in the brain was important for reversing memory deficits in an Alzheimer's disease model. It is further shown that m266 can be detected in the brain in the high-dose groups whereas plasma increase is detected in all groups. Fig. 4D of Dodart et al. again showed that there is direct evidence for the presence ofm266 in the brain. This is also confirmed in the second paragraph on page 455, left column of Dodart et al., where it is stated that the antibody/Aß complex was also found in CSF at the lowest dose found to be effective in reversing learning and memory impairment . This again shows that besides the detectability problem this group had -the failure to detect m266 does not mean that there is no antibody present) - one would see the action of a peripherally administered anti Aβ antibody such as m266 (or of two additional examples: m3D6 and m10D5 also mentioned in DeMattos, left column, third paragraph, last sentence) as being able to cross the BBB and have a direct effect on Aβ/Aβ deposits in the brain.

8. It is therefore clear that, in contrast to the interpretation of the USPTO, administration of the m266 antibody is not connected to an exclusively peripheral effect but that this antibody is present in the brain and its extra-cellular compartment (in the CSF) *in vivo* following peripheral administration. Indeed, other antibodies for which crossing of the BBB is known [see Kotilinek et al (J. Neuroscience 22 (2002), 6331-6335), Bard et al. (Nat. Med. 6 (2000), 916-.919),,and, Hokoshi et al. (BBRC 325 (2004), 384-387] exert effects in the brain.

9. It is also known that an antibody in the CNS can foster the release of Aβ from the CNS significantly (see DeMattos., page 8852; and the attached Kotilinek et al, Bard et al, Hokoshi et al and Wilcox et al. (J. Neuroscience 23 (2003), 3745-3751) showing the crossing of peripherally administered anti-Abeta-antibodies over the BBB and the presence and

functionality of such antibodies in the CSF/brain). Accordingly, the finding of DeMattos is not teaching that peripheral presence of an Aβ antibody (an APP antibody; i.e. an amyloid binding agent) is the basis for the effects observed *in vivo*.

10. Moreover, it has to be acknowledged that the velocity of Aβ release into the plasma is, in principle, also based on the enhanced presence of high affinity binders in the CNS following application. It is well known to the expert in the field that antibodies to CNS proteins reach the brain very fast after peripheral application (one hour or less) and can thus exert their function *in situ* very early after administration. See the attached publications of Freund (J. Exp. Med. 51 (1930), 889-902) and Masliah et al. (Neuron 46 (2005), 857-868).

11. To interpret the data contained in DeMattos, one would also have to include the extra-neuronal sources of Aβ which are present in the periphery of AD patients (as well as in the transgenic animals in the DeMattos study).

12. The blood and its cellular components are an important source for amyloid peptides. Platelets are well-known to constitute one of the major sources of Aβ in the blood. The antibody presence in the blood can thus also lead to a dramatic release from Aβ from the platelets into the bloodstream accounting at least, in part, for the increase of Aβ reported by DeMattos. Accordingly, the direct contact of high antibody levels with platelets is how the data presented by DeMattos can be reasonably interpreted.

13. In addition it is known that the highly abundant plasma protein albumin is capable of binding the amyloid peptide and is consequently usually associated with Aß peptides in the circulation. In fact, almost 90% of amyloid peptides in plasma are bound to albumin (see attached article of Biere et al., JBC 271 (1996), 32916-32922).

14. The USPTO on page 5, 2nd paragraph of the paper dated May 19, 2010 stated that "the ability to bring Aβ across the membrane is unique to this antibody and it can pull Aβ out of the brain." However, this conclusion is not correct, and even if it was correct this still does

not suggest to one in this field to do what is defined by the method of claim 1 with a reasonable expectation of success.

15. First, DeMattos clearly reports that the effect according to Fig. la is also present for other antibodies including m3D6 and m10D5 (see page 8851, left column, 3rd paragraph, last sentence). Moreover, a small amount of the antibody in the brain is sufficient to reduce the very lowly concentrated toxic Aβ species at synapses responsible for disturbing memory formation as described by the article of Dodart et al. (the same group that published DeMattos). As monoclonal antibodies are known to cross the BBB, albeit in low amounts of about 1 to 5%, even this amount is sufficient to clear amyloid deposits during chronic treatment. Moreover, cognitive benefits are detectable before amyloid reduction is detectable in the brain in the model used. This shows that even for DeMattos, one would understand that the effects reported by DeMattos relate to an effect of the antibody in the brain as being important for the overall results.

16. Even if it is true that Fig. 1 A would imply to a person skilled in the art that apheresis with an anti-Aβ antibody would decrease the concentration in the compartment on the other side of the membrane, in the case of the apheresis according to this application, this other side of the membrane would be the human blood and not the human brain. Moreover, it was not shown by DeMattos that the mere reduction of plasma level of Aβ was then sufficient to reduce Aβ in the brain. This is an oversimplification artificially pulled out from this article by the USPTO with the knowledge of having read this application because I certainly do not come to that conclusion when reading DeMattos. In fact, in 2001, DeMattos with the knowledge of the subsequent work of this group, see again Dodart et al, one in this field would know that in addition to the known effects that administration of an antibody in the blood system has in the clearance of the compound to which this antibody binds (i.e. to Aβ) this antibody also exerts direct effects in the brain. This was regarded as essential for the

effect of the administration of this antibody to the mouse model. Although being administered to the periphery, the antibody evidently crosses the BBB and has effects there which were decisive for the overall effect reported (see Dodart et al. 2002).

17. Kojima shows that an increased plasma level of p2-MG can be reduced in hemodialysis patients (see table 1 of Kojima). However, this reduced level in HD patients is still significantly higher than normal levels (see also table 1 of Kojima). IgE or SAP levels in plasma are shown to be decreased by applying the method. However, Kojima does not contain any information that amyloid depositions in certain compartments or even in the brain decrease with this method. Even if Kojima had shown reduction of several amyloid depositions, this would still only be a reduction within the blood system, i.e., in the periphery.

18. Claim 1 of this application fundamentally differs from Kojima's approach, because apheresis is applied to the blood or plasma flow to reduce amyloid deposition, including APP, in the brain (and not in plasma). Plasma of AD patients does not show an elevated Aβ level. Quite in contrast, DeMattos shows a rapid 1000-fold increase in plasma Aβ. Even though, a corresponding β2-MG antibody has not been tested in Kojima, it would be expected that such rapid increase in β2-MG does not occur, because the biology of the proteins in Kojima differs so fundamentally from Aβ used in the context of this application.

19. It is clear to me that DeMattos differs fundamentally from the manner and goal that claim 1 of this application sets out to treat AD and the addition of Kojima simply is not combinable with DeMattos. Even if one did combine those references, there would not have been a reasonable expectation of success due in large part to the significant and fundamental differences between β2-MG as taught in Kojima compared to the Aβ in the claims of this application.

20. The undersigned declares further that all statements made herein are of his own knowledge, are true and that all statements made on information are believed to be true. Further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

_____
Eliezer Masliah, M.D.

October 24, 2010
Date

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Eliezer Masliah, M.D.	Professor of Neurosciences and Pathology
eRA COMMONS USER NAME Masliah	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
National Institute of Pediatrics, Mexico City, Mexico		1982-1983	Soc. Serv. in Pediatric Pathology
National Autonomous University of Mexico City, Mexico	M.D.	1983	Medicine

A. Personal Statement

The main objectives of the research at my laboratory of Experimental Neuropathology at UCSD are to better understand the molecular mechanisms of synaptic pathology in neurodegenerative disorders and to develop new experimental therapies for Alzheimer's Disease and Parkinson's Disease. Alterations in the rate of *aggregation*, *synthesis* or *clearance* can result in the formation of toxic oligomers, neurodegeneration and further propagation of the disease. For this reason efforts in my laboratory have been directed at better understanding the mechanisms of oligomers formation and clearance of Abeta and synuclein. Therefore, strategies directed at promoting clearance of oligomers might be of therapeutic value for Alzheimer's Disease and Parkinson's Disease.

Positions and Honors

Positions and Employment

1983-1986 Resident, Anatomic Pathology, National Institute of Nutrition, Mexico City, Mexico
1988-1990 Postdoctoral Fellow, Pathology of Neurodegenerative Diseases, University of California San Diego, La Jolla, California
1990-1991 Assistant Research Neuroscientist, University of California San Diego, La Jolla, California
1991-1993 Assistant Professor, Departments of Neurosciences and Pathology, University of California San Diego, La Jolla, California
1993-1996 Associate Professor, Departments of Neurosciences and Pathology, University of California San Diego, La Jolla, California
1997-present Professor, Departments of Neurosciences and Pathology, University of California San Diego, La Jolla, California
1995-present Director of Autopsy Service, Department of Pathology, University of California San Diego, La Jolla, California

Other Experience and Professional Memberships

1990- Member, American Association of Neuropathologists
1995- Member, Society for Neurosciences
1996-00 Member, NIH Neuroscience of Aging Review Committee
2007- Member, NIH Cellular and Molecular Biology of Neurodegeneration Study Section
2010- Member, Medical and Scientific Advisory Council of the Alzheimer's Association

Honors

1988 Weil Award (Honorable Mention), American Association of Neuropathologists
1989 Alzheimer Association/George F. Berlinger Faculty Scholar Award
1990 Weil Award (Honorable Mention), American Association of Neuropathologists

2006 MERIT Award NIH-NIA
2010 The Dorothy Russell Memorial Medal, British Neuropathology Society

B. Selected peer-reviewed publications (Selected from 454 peer-reviewed publications).

Most relevant to the current application

1. **Masliah** E, Rockenstein E, Adame A, Alford M, Crews L, Hashimoto M, Lee M, Chilcote T, Games D, Schenck D. Effects of α-synuclein immunizations in a mouse model of Parkinson's disease. Neuron 46:857-68, 2005. PMID: 15953415
2. Uehara T, Nakamura T, Yao D, Shi ZQ, Gu Z, Ma Y, **Masliah** E, Nomura Y, Lipton SA. S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. Nature 441:513-7, 2006. PMID: 16724068
3. Tsigelny IF, Crews L, Desplats P, Shaked GM, Sharikov Y, Mizuno H, Spencer B, Rockenstein E, Trejo M, Platoshyn O, Yuan JX, **Masliah E**. Mechanisms of hybrid oligomer formation on the pathogenesis of combined Alzheimer's and Parkinson's Disease. PLoS ONE. 9:e3135, 2008. PMID: 18769546
4. Desplats P, Lee HJ, Bae EJ, Patrick C, Rockenstein E, Crews L, Spencer B, **Masliah E**, Lee SJ. Inclusion formation and neuronal cell death through neuron-to neuron transmission of alpha-synuclein. Proc Natl Acad Sci U S A. 106(31):13010-5, 2009. PMID: 19651612
5. Spencer B, Potkar R, Trejo M, Rockenstein E, Patrick C, Gindi R, Adame A, Wyss-Coray T, **Masliah E**. Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body disease. J Neurosci. 28;29(43):13578-88, 2009. PMID: 19864570

Additional recent publications of importance to the field (in chronological order)

1. Rockenstein E, Torrance M, Adame A, Mante M, Bar-on P, Rose JB, Crews L, **Masliah** E. Neuroprotective effects of regulators of the glycogen synthase kinase-3beta signaling pathway in a transgenic model of Alzheimer's disease are associated with reduced amyloid precursor protein phosphorylation. J Neurosci 27:1981-91, 2007. PMID: 18627032
2. Tsigelny IF, Bar-On P, Sharikov Y, Crews L, Hashimoto M, Miller MA, Keller SH, Platoshyn O, Yuan JX, **Masliah** E. Dynamics of alpha-synuclein aggregation and inhibition of pore-like oligomer development by beta-synuclein. FEBS J 274:1862-77, 2007. PMID: 17381514
3. Belinson H, Lev D, **Masliah** E, Michaelson DM. Activation of the amyloid cascade in apolipoprotein E4 transgenic mice induces lysosomal activation and neurodegeneration resulting in marked cognitive deficits. J Neurosci 28:4690-701, 2008. PMID: 18448646
4. Crews L, Mizuno H, Desplats P, Rockenstein E, Adame A, Patrick C, Winner B, Winkler J, **Masliah** E. Alpha-synuclein alters Notch-1 expression and neurogenesis in mouse embryonic stem cells and in the hippocampus of transgenic mice. J Neurosci 16:4250-60, 2008. PMID: 18417705
5. Pickford F, **Masliah** E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, Small S, Spencer B, Rockenstein E, Levine B, Wyss-Coray T. The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. J Clin Invest 118:2190-99, 2008. PMID: 18497889
6. Marongiu R, Spencer B, Crews L, Adame A, Patrick C, Trejo M, Dallapiccola B, Valente EM, **Masliah E**. Mutant Pink1 induces mitochondrial dysfuntion in a neuronal cell model of Parkinson's disease by disturbing calcium flux. J Neurochem. 6:1561-74, 2009. PMID: 19166511
7. Nagahara AH, Merrill DA, Coppola G, Tsukada S, Schroeder BE, Shaked GM, Wang L, Blesch A, Kim A, Conner JM, Rockenstein E, Chao MV, Koo EH, Geschwind D, **Masliah E**, Chiba AA, Tuszynski MH. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. Nat Med. 3:331-7, 2009. PMID: 19198615
8. Rose JB, Crews L, Rockenstein E, Adame A, Mante M, Hersh LB, Gage FH, Spencer B, Potkar R, Marr RA, **Masliah E.** Neuropeptide Y fragments derived from neprilysin processing are neuroprotective in a transgenic model of Alzheimer's disease. J Neurosci. 4:1115-25, 2009. PMID: 19176820
9. Shankar GM, Leissring MA, Adame A, Sun X, Spooner E, **Masliah E**, Selkoe DJ, Lemere CA, Walsh DM. Biochemical and imunohistochemical analysis of an Alzheimer's disease mouse model reveals the presence of multiple cerebral Abeta assembly forms throughout life. Neurobiol Dis. 36:293-302, 2009. PMID: 19660551

10. Cohen E, Paulsson JF, Blinder P, Burstyn-Cohen T, Du D, Estepa G, Adame A, Pham HM, Holzenberger M, Kelly JW, Masliah E and Dillin A. Reduced IGF-1 Signaling Delays Age-associated Proteotoxicity in Mice. Cell. 139:1157-69, 2009. PMID: 20005808

C. Research Support

Ongoing

2 P50 AG05131 (Galasko, Douglas) 05/01/2004 - 03/31/2014
NIH
Alzheimer's Disease Research Center - D: Neuropathology Core / 2:Testing the Role of Transport Disturbance in AD
The major goal focuses on early diagnosis and tracking of Alzheimer's disease as well as potential mechanisms of neurodegeneration and repair.
Role: PI-Neuropathology Core

2 P01 DA12065 (Grant, Igor) 05/15/2005 – 04/30/2010
NIH
Neuroaids: Effects of Methamphetamine and HCV – 6: Neurobiology
The major goal of this project is to examine neuropathological alteration and patterns of neurodegeneration associated with methamphetamine use in the context of HIV infection.
Role: PI – Neurobiology Core

5 R01 MH073510 (McGrath, Michael) 02/14/2005 – 01/31/2010
NIH/University of California, San Francisco
HIV Sequence Evolution in AIDS Dementia
The major goal is to investigate the contribution of macrophage subpopulation to the neurodegenerative process in HAD.
Role: PI – Subcontract

1 R01 NS050041 (Gonzalez, Gilberto) 03/01/2005 – 02/28/2010
NIH/Mass General Hosp
Advanced MR Spectroscopy of SIV Brain Injury
The major goal of this grant is to assess the neurological and neuron damage as well as quantification of macrophages in the brain.

5 P30 MH62512 (Grant, Igor) 04/01/2006 – 03/31/2011
NIH
HIV Neurobehavioral Research Center – C: Neurobiology Core
The major goal is to examine the development and course of neurocognitive & neuropsychiatric consequence of HIV disease.
Role: PI – Neurobiology Core

1 R01 MH79881-01 (Everall, Ian) 09/27/2006 – 07/31/2011
NIH
Somatostain & Stress-Related Genes in HIV & Comorbid Major Depressive Disorder
To determine the biological basis for co-occurance of major depressive disorder in the setting of HIV infection based on brain gene expression, molecular and cellular pathology.
Role: Co-Investigator

5 R37 AG18440 (Masliah, Eliezer) 09/01/2001 – 08/31/2011
NIH
Alpha-Synuclein Clearanc Strategies as a Treatment for Lewy Body Disease
The major goal of this project is to ascertain the anti-aggregations potential of α-synuclein as a therapeutic target for the development treatment of Parkinson's disease.

Role: PI

5 P30 NS057096 (Lipton, Stuart) 09/01/06 – 08/31/2011
NIH/Burnham Institute
The major goal of this project is to provide to investigators in the La Jolla Neurosciences community a set of technical and neurobiological resources that allows analysis of the degenerative processes associated with neurological conditions.
Role: PI of Neuropathology Core

5 R01 MH62962 (Masliah, Eliezer) 04/01/2007 – 03/31/2012
NIH
Mechanisms of Neuroprotection in HIV Encephalitis
The major goals are to determine patterns expression in the brains of AIDS patients are associated with neuroprotection vs. neurodegeneration.
Role: PI

5 R37 AG11385 (Mucke, Lennart) 06/15/2002 – 03/31/2012
NIH/Gladstone Inst
Transgenic Models to Study Alzheimer's Disease
The major goal is to develop new transgenic models of Alzheimer's disease to investigate amyloid toxicity.
Role: PI – Subcontract

CURRICULUM VITAE

Eliezer Masliah, M.D.

Date of Birth: December 9, 1958, Mexico City, Mexico
Residence Status: U.S. Citizen
Marital Status: Married
Address: 4661 Corte Mar Del Corazon, San Diego 92130

Education:

1977 - 1982 **National Autonomous University of Mexico** Mexico City, Mexico
School of Medicine
Doctor of Medicine

1982 - 1983 **National Institute of Pediatrics** Mexico City, Mexico
Social Service in Pediatric Pathology

Residencies:

1983 - 1984 **National Institute of Nutrition** Mexico City, Mexico
General Pathology

1984 - 1985 **National Institute of Cancerology** Mexico City, Mexico
Oncologic Pathology

1985 - 1986 **National Institute of Pediatrics** Mexico City, Mexico
Pediatric Pathology

Postdoctoral Training:

1988 - 1990 **University of California, San Diego** La Jolla, California
Department of Neurosciences
Pathology of Neurodegenerative Diseases

Academic Appointments:

1990 - 1991 **University of California, San Diego** La Jolla, California
Department of Neurosciences
Assistant Research Neuroscientist

1991 - 1993 **University of California, San Diego** La Jolla, California
Departments of Neurosciences and Pathology
Assistant Professor in Residence

1993 - 1994 **University of California, San Diego** La Jolla, California
Departments of Neurosciences and Pathology
Associate Professor in Residence

1994 - 1997 **University of California, San Diego** La Jolla, California
Departments of Neurosciences and Pathology

Associate Professor (Tenured Track)

1997 - **University of California, San Diego** — La Jolla, California
Departments of Neurosciences and Pathology
Professor

1995- **University of California, San Diego** — San Diego, California
Medical Center, Department of Pathology
Director of Autopsy Service

Board Certifications:

1986 Mexican Board of Anatomic Pathology
1989 Anatomic Pathology Specialty
National Autonomous University of Mexico — Mexico City, Mexico

License:

Department of Public Education — Mexico City, Mexico
General Department of Professions, License No. 873217

Medical Board of California — California, USA
State of California, License No. A067390

Laboratory Experience:

1979 - 1986 **National Autonomous University of Mexico** — Mexico City, Mexico
School of Medicine
Laboratory Instructor in Human Histology

1987 - 1988 **University of California, San Diego** — La Jolla, California
Department of Neurosciences
Laboratory Assistant in Neuropathology

Teaching Experience:

1979 - 1986 **National Autonomous University of Mexico** — Mexico City, Mexico
School of Medicine
Laboratory Instructor in Histology, Embryology and Genetics

1990 - 1995 **University of California, San Diego** — La Jolla, California
Department of Neurosciences
Instructor in Mammalian Neuroanatomy Laboratory (SOM256)

1992 - 1998 **University of California, San Diego** — La Jolla, California
Department of Pathology
Instructor in Neuropathology (portion of SOM208)

1997 - **University of California, San Diego** — La Jolla, California
Department of Pathology
Instructor (portion of Path221 & PTH220)

Scientific Associations:

American Association of Neuropathologists

Society for Neurosciences

Honors and Awards:

1989 Honorable Mention for the Weil Award. American Association of Neuropathologists

1990 Alzheimer Association/George F. Berlinger Faculty Scholar Award

1993 Honorable Mention for the Weil Award. American Association of Neuropathologists

Other:

1992 - present Ad Hoc Reviewer NINDS/NIH

1996 - present Member, Neuroscience of Aging Review Committee, NIA/NIH

1996 - 2000 Associate Editor, Neuroscience Letters

1998 – present Member, Editorial Board, Journal of Neuropathology & Experimental Neurology

1998 – present Member, Editorial Board, Laboratory Investigation

1999 – present UCSD, Cadaver use advisory review committee

2001 – present UCSD, Dean's SOM Space Advisory Committee

2002 – present UCSD, Academic Senate

2002- present ADRC/UCI Advisory Board Committee

2002- present ADRC/UCSF Advisory Board Committee

2004 – present UCSD, Search Committee, Department of Pathology

2006 – present UCSD Committee on Research

Research Support:

Completed

Funding Agency	Grant/Award Number	Project Title	Award Period	Yearly Direct Costs
NIH/NIA	1 RO1 AG8201 (PI-Minority investigator research supplement initiative)	Structure and function in Alzheimer Disease	1/1/90-3/31/93	268,584
NIH/NIA	1 RO1 AG10689 (PI)	Subcellular basis of synaptic pathology Alzheimer disease	9/29/91-6/30/94	242,003

Alzheimer Assoc.	FS-90-008 (PI)	Structural and quantitative synaptic changes in relation to amyloid Alzheimer disease	1/1/92-12/31/93	40,835
Calif. State, Dept. Of Health	92-15936 93-18642 94-20619 (PI)	Role of amyloid precursor protein in the synaptic organization of the Neocortex in transgenic mouse models	12/1/92-11/30/95	197,916
Am. Health Assist. Foundation	---- 81,911 (CO-PI)	Cloning of a novel protein of AD amyloid		4/1/93-3/31/94
NIH/NINDS	RO1 NS33056 (PI-subcontract Gladstone Institute)	In vivo CNS effects of HIV coat and regulatory proteins	4/1/94-2/28/98	70,694
NIH/NIA	5 P50 AGO5131 (PI-Project 4)	Alzheimer's Disease Research Center (Synaptic Alterations in APP Transgenic Mice)	4/1/94-3/31/96	134,958
NIH/NIA	5 P50 AGO5131 (CO-PI Neuropath Core)	Alzheimer's Disease Research Center	4/1/94-3/31/99	824,452
NIH/NIMH	5 P50 MH45294 129,445 (PI Neuropath Core)	HIV Neurobehavioral Research	6/1/94-11/30/95 12/1/95-11/30/00	 571,567
Athena Neurosciences/ Lilly Pharmaceutical	---- 23,179 (PI)	Ultrastructural alterations in PDAPP tg mouse		7/1/94-12/31/96
NIH/NIA	2 RO1 AG10689-S1 (PI-supplement)	Subcellular basis of synaptic pathology in Alzheimer's disease	9/30/94-6/30/96	109,083
NIH/NINDS (Gladstone Inst)	RO1 NS34602 (PI-subcontract)	Transgenic models to study AIDS/dementia complex	7/1/95-5/31/99	93,686
EBEWE-Arzneimittel	--- 164,485 (PI)	Neuroprotective role of Cerebrolysin in transgenic Models of Alzheimer's disease		12/01/95-5/14/99
Alzheimer's Assoc.	IIRG-95-007 150,000 136,365(PI)	ApoE role in synaptogenesis and neurodegeneration in Alzheimer Disease		7/1/96-6/30/98

Ruth K. Broad Fdn (Duke University)	---- (PI)	Synaptotrophic effects of ApoE	7/1/96-6/30/98	94,090
NIH (Massachusetts General Hospital)	1 RO1 NS34626 198,802 (PI-subcontract)	Molecular neuroimaging of AIDS dementia: animal models		1/1/97-6/30/99
Athena Neurosciences	---- 31,735	Ultrastructural alterations in PD APP mouse		1/1/97-12/31/98
NIH/NIA	2 RO1 AG10689 (PI)	Subcellular basis of synaptic Pathology in Alzheimer disease	1/1/97-11/30/01	549,391
NIH/NIA	5 RO1 AG05131 (PI-supplement Project 4)	Alzheimer's Disease Research Center (Synaptic alterations in APP transgenic mice)	4/1/97-3/31/99	248,488
NIH/NIA	5 P50 AG05131 (PI-pilot project)	Molecular rearrangements of alpha-1 antichymotrypsin and their effects on biological functions of serpin: relevance to Alzheimer's disease pathogenesis	4/1/97-3/31/98	18,000
Stein Inst. (UCSD)	--- (PI)	Novel role of RAP in neuro-transmitter modulation: implications for aging and AD	7/1/97-6/40/98	10,000
NIH/NIMH (Scripps)	RO1 MH57061-02 (PI-subcontract)	Borna disease virus and neuropsychiatric disorders	12/1/97-11/30/00	60,000
NIH/NIMH	R24-MH59745 (PI-Neuropathology Unit)	California NeuroAIDS Tissue Network	9/30/98-9/29/03	653,095
NIH/NIDA	P01-DA 12065 505,005 (PI-Project 4)	Mechanisms of Neurodegenera-tion in METH dependent HIV+ Positive		4/1/99-3/31/04
NIH (Massachusetts General Hospital)	R01 RR13213 (PI-subcontract)	Advanced MR spectroscopy of SIV brain injury	12/1/97-11/30/02	60,000
NIH/NIA	5 P50 AGO5131	Alzheimer's Disease Research	4/1/99-3/31/04	370,000

	(PI-Project 4)	Center (NACP Transgenic Mice)		
NIH/NIA	5 P50 AGO5131 (CO-PI Neuropath Core)	Alzheimer's Disease Research Center	4/1/99-3/31/04	625,000
NIH/NINDS (Gladstone Inst)	AG11385 (PI-subcontract)	Transgenic models of Alzheimer's disease	4/1/98-3/31/99	156,783
NIH/NIA (Supplement)	3 RO1 AG11385 (PI-subcontract Gladstone Institute)	Transgenic models to study Alzheimer's disease	10/1/96-6/30/97	50,002
NIH/NINDS (Gladstone Institute)	MH58164-02 (PI-subcontract)	Therapeutic targets in tg mice/Inhibition of HIV neurotoxicity in tg mice	7/1/97-6/30/01	120,000
MJ Fox Foundation	(PI)	Transgenic models for treatment of Park Dis	6/1/01-5/31/02	50,000
Proteotech	(PI)	Anti-amyloid.... in APP tg models	1/1/01-5/30/01	50,000
NIH	5 R01 AG10689-08 (PI)	Basis of Synaptic Pathology in Alzheimer's disease	1/1/97-11/30/02	111,809
NIH	5 R01 NS34626-07 (PI)	Molecular Neuroimaging of AIDS Dementia: Animal Models	5/1/99-4/30/03	19,161

Current

Funding Agency	**Grant/Award Number**	**Project Title**	**Award Period**	**Yearly Direct Costst**
NIH	5 P30 MH62512 (PI -Neurobiology Core)	HIV Neurobehavioral Research Center - CC: Neurobiology Core	04/01/06 – 03/31/11	$177,825
NIH	2 R24 MH59745 (PI - Project 8)	NeuroAIDS Tissue Network	09/30/98 - 05/31/03	$131,482
NIH	5 R37 AG18440 (PI)	Alpha-synuclein clearance strategies as a Treatment for Lewy Body Disease	09/01/01 - 08/31/11	$250,000
NIH	5 R01 MH62962 (PI)	Mechanisms of Neuroprotection in HIV Encephalitis	02/20/02 – 03/31/12	$250,000
NIH (Gladstone Inst)	5 P01 AG022074 (PI- Subcontract)	Proteopathies of the Aging Central Nervous System	06/15/03 - 05/31/08	$144,4810
NIH (Gladstone Inst)	5 R37 AG11385 (PI - Subcontract)	Transgenic Models to Study Alzheimer's Disease	06/15/02 - 03/31/12	$90,472

NIH (Salk Institute)	5 P01 AG10435 (PI- Subcontract)	Lentivirus for Modeling and Treatment of AD in TG Mice	09/30/02 - 08/31/07	$157,345
NIH	5 P01 NS044233 (Co-PI - Project 3)	Pathogenesis and Diagnosis of Multiple System Atrophy-Project 3	09/30/03 - 06/30/08	$144,481
Elan Pharmaceuticals Inc	PI	Development of an Anti-Parkinson's Vaccine in Transgenic Mice	04/01/04 - 03/31/08	$80,000
NIH	2 P50 AG05131 (PI - Project 4)	Alzheimer's Disease Research Center - D: Neuropathology Core/2: Testing the Role of Transport Disturbance in AD	05/01/04 - 03/31/09	$206,817
NIH	2 P01 DA12065 (PI-Neuropath)	NeuroAIDS: Effects of Methamphetamine - Neuropathology/ Neuropathology Supplement	07/01/04 - 06/30/09	$108,951
US-Israel Binational Science	PI	Isoform Specific Effects of Apolipoprotine E on the Aggregation, Deposition and Toxicity of Beta-amyloid in vivo	10/01/04 - 09/30/08	$10,000
Stanford University/ Larry Hillblom Foundation	PY-2598 (PI - Subcontract)	Synaptic Stucture and Function in Down Syndrome	01/01/05 - 12/31/06	$22,000
NIH (University of California, San Francisco)	5 R01 MH073510 (PI- Subcontract)	HIV Sequence Evolution in AIDS Dementia	02/14/05 - 01/31/10	$250,000
Elan Pharmaceuticals Inc	PI	Role of Mononuclear Leukocytic Infiltration in the Early Neuro-Inflammatory Response in Patients with Alzheimer's Disease and Lewy body dementia	07/01/05 - 06/30/06	$70,000
UC Discovery Grant / Kinexis Inc.	bio05-10557 (PI – Subcontract)	Alzheimer's vaccine that avoids inflammatory side effects.	09/01/06 – 08/31/08	$75,000
Michael J. Fox / Proteotech	(PI-Subcontract)	New small molecule inhibiting agents of alpha-synuclein and Lewy body formation as disease-modifying treatments for Parkinson's disease	01/01/06-12/31/08	$96,000
NIH (Mass General Hospital)	(PI-Subcontract)	Advanced MR Spectroscopy of SIV Brain Injury	03/01/05 –02/28/10	$217,469

List of Students:

Pathology Residents:

Jennifer Strother	1996-97
Warren Snider	1996-
Barney Welsh	1996-
TJ Bisla	1996-
Swen Holland	1996-97
Harper Summers	1996-
Cathy Palmer	1997
Siria Poucell	1997;
Vivian Tan	1997-
Slav Uglik	1997-98
Peilin Zhang	1997-98
Min Huang	1997-98
Phil Cacheris	1998
Serguei Bannykh	1998-02
Wenyi Wang	1998-99
Hongchen Jia	1998-99
Mark Lu	1998-02
Dawn Jacobson	1998-02
Galina Bennykh	1999-02
Lisa Albers	1999-02
Michan Afsari	1999-02
Keith Overstreet	2000-02
Helena Whang	2000-02
Shawn Emery	2000-01
Emma Du	1999-02
Kelli Hanson	1999-02
Anna Moyahan	2000-01
Chris Wixom	2000-01
Chris Holt	2001-02
Joseph Hughes	2001-02
Linda Roley	2001-02
Ayce Unal	2001-02
Truc Pham	2002-03; 05
Mark Butler	2002-03;
Omid Bakhtar	2002-03;
Nicole Nilson	2002-03; 05
Carter Wahl-	2002-03;
Chris Wixom	2002-03
Susan Duncan	2002-03
Joan Bernard	2003-04
Joe Hughes	2003-04
Arman Kasian	2003-04
Grace Lin	2003-04
Mike Peterson	2003-04
Curtis Strong	2003-04
Suchi Pandey	2003-04
Kyle Noskoviak	2003-04
Mark Peterson	2003-04
Owan Chan	2003-04
Sima Fargahi	2004-05
Ryan Olson	2004-05
Richard Scuder	2005-05

Graduate students:

Karen Huang	01/94 -
David Kang	04/94 -
Silvia Tara	06/95 -
Emily Van Uden	04/96 –
Christine McGiffert	09/96 -
Lynn Weber	09/96 -
Sarah Moskowitz	01/97 -
Helen Le-Nicolescu	01/97 -
Dan Kalafus	01/97 -
Aaron Bowman	01/97 -
Keith Magert	01/97 -
Jason Greenwald	01/97 -
Leigh Hsu	06/96 -
Isaac Veinbergs	06/96 -
Kelly Chang	01/99 -
David Merriam	04/99 -
David Moore	03/01 -
Jon Violin	10/99
Gudren Wakonning	02/99-
Jason Freshwater	04/00
Dan Oral	02/00
Ruth Carper	08/00
Paula Soto	02/01 -
Brian Kasper	07/01
Zhen Li	05/01 -
Laura Rassor	11/01
Jorge Munera	08/05 –
Leslie Crews	09/05 -
Erin Conn	06/06 –
Roberta Marongiu	04/06 –

Undergraduate students:

Robert Johnson	04/91 -
Archana Jaiswal	04/92 -
Isaac Veinbergs	04/92 -
Mitchell Voight	04/92 -
Mario Cole	10/92 -
Karina Herrera	07/93 -
Mark Smith	02/94 -
Shelly Banerjee	06/94 -
Julio Diaz	06/94 -
Mike Mante	01/95 -
Lon Tran	04/95 -
Laura Fox	08/95 -
Abraham Hanono	10/95 -
Arie Yafeh	03/96 -
Mario Manese	04/97 -
Jennifer Mattli	06/98 -
Michael Kim	01/99 -
Gabriel Larrea	12/00 –
Matthew Izu	07/02 –
Timothy Wilson	4/05-6/05

Medical Students:

Lida Ghaderi	09/91 -
Matt Kodsi	05/91 -
John Leake	10/92 -
Ivan Burgos	05/93 -
Elana Lehman	04/94 -
Ji Yoo	09/95 -
Mark Tiffany	03/96 -
Shawn Emery	09/99 -
Patricia Hacket	09/99 -
Jason Himmel	07/01 –
Brian Webster	06/03 –
Jennifer Kum	01/04 –
Jason Tovar	6/02 –
Kathy Rameriz	02/03-
Geoffrey Biard	05/03-
Mike Cho	06/03-

MD/PhD Students:

Peter Nakaji	10/92 -
Matt Kodsi	04/95 -
Albert Lai	01/95 -

Post-doctoral Fellows:

Nianfeng Ge	06/93 -
Virginia Sanders	
Shi Li	01/96 -
William Samuel	04/95 -
Veronika Thorns	06/96 -
Makoto Hashimoto	05/96 -
Gilbert Ho	09/98 –
Takato Takenouchi	12/98 -
Ayako Takeda	08/97 -
Dianne Langford	06/00 -
Ingrid Lang	06/98 -
David Song	07/01 -
Dalen Agnew	11/01 -
Jeannelyn Estrella	06/05 –
Pazit Bar-On	09/02 –
Aline Grigorian	09/02 –
Hideya Mizuno	11/04 –
Gideon Shakked	07/06 -
Shigeto Sato	10/06 -
Andrew Koob	01/07 -
Paula Desplats	05/07 -

Visiting Scholars:

Koichi Wakabayashi	1994
Ian Everall	1996 - 97,
Federico Licastro	1996 - 97,
Jack de la Torre	1999 - 00
Gudrun Wakonigg	1998 - 99
Ingrid Lang	1999
Seigo Tanaka	1999 - 00
Koichi Kawahara	2003 -
Phil Hyu Lee	2007 -

PUBLICATIONS:

Professional Thesis:

1. For the MD Degree: "Identification of GFAP in tumors of the nervous system in children". National Institute of Pediatrics and National Autonomous University of Mexico, Mexico City, Mexico. 1982
2. For the Pathology Specialty: "Morphometric Studies in Pathobiology". National Institute of Nutrition/ National Autonomous University of Mexico, Mexico City, Mexico. 1987

Research Articles:

1981-1987

1. Reyes M, **Masliah** E, Besthoff L. [Neuroblastoma. Recent Concepts.] Rev Fac Med Mex 24:19-34, 1981 (Spa)
2. **Masliah** E, Perez-Tamayo R. [A Note on the Histopathology of Invasive Amebiasis of the Large Bowel.] Patologia (Mex) 22:233-45, 1984. (Eng abstract). (Spa)
3. **Masliah** E, Perez-Tamayo R. [Submassive Hepatic Necrosis. Quantification of the hepatic damage and correlation with clinical data.] Patologia (Mex) 23:39-46, 1985. (Eng abstract) (Spa)
4. **Masliah** E. A unified hypothesis for the origin of soft tissue sarcomas of unknown origin. Med Hypoth 20:393-401, 1986.
5. **Masliah** E. [A new thermodynamic theory to explain life under normal and pathological conditions.] Patologia (Mex) 24: 79, 1986 (Letter) (Spa).
6. Mora–Tiscareno A, **Masliah** E, Gonzalez J, Ramirez J, Meneses A. [Cervico–uterine carcinoma: study of 56 autopsy cases]. Rev INC (Mex) 33:470–8, 1987 (Eng abst) (Spa).

1989-1990

7. Cole G, **Masliah** E, Huynh TV, DeTeresa R, Terry RD, Okuda C, Saitoh T. An antiserum against amyloid β–protein precursor detects a unique peptide in Alzheimer brain. Neurosci Lett 100:340–6, 1989. [PMID: 2503790]
8. **Masliah** E, Terry RD, Buzsaki G. Thalamic nuclei in Alzheimer disease: evidence against the cholinergic hypothesis of plaque formation. Brain Res 493:240–6, 1989. [PMID: 2765898]
9. Hansen L, **Masliah** E, Terry RD, Mirra SS. A neuropathological subset of Alzheimer's disease with concomitant Lewy body disease and spongiform change. Acta Neuropathol 78:194–201, 1989. [PMID: 2546359]
10. **Masliah** E, Terry RD, DeTeresa R, Hansen LA. Immunohistochemical quantification of the synapse–related protein synaptophysin in Alzheimer disease. Neurosci Lett 103:234–9, 1989. [PMID: 2505201]
11. **Masliah** E, Terry RD, Alford M, DeTeresa R. Quantitative immunohistochemistry of synaptophysin in human neocortex: an alternative method to estimate density of presynaptic terminals in paraffin sections. J Histochem Cytochem 38:837–44, 1990. [PMID: 2110586]
12. Iimoto DS, **Masliah** E, DeTeresa R, Terry RD, Saitoh T. Aberrant casein kinase II in Alzheimer disease. Brain Res 507:273–80, 1990. [PMID: 2110586]

13. Hansen L, Salmon D, Galasko D, **Masliah** E, DeTeresa R, Thal L, Katzman R, Pay M, Hofstetter R, Klauber M, Rice V, Butters N, Alford M. The Lewy body variant of Alzheimer's disease: a clinical and pathological entity. Neurology 40:1–8, 1990. [PMID: 2153271]
14. **Masliah** E, Cole G, Shimohama S, Hansen L, DeTeresa R, Terry RD, Saitoh T. Differential involvement of protein kinase C isozymes in Alzheimer's disease. J Neurosci 10:2113–24, 1990. [PMID: 2376771]
15. **Masliah** E, Galasko D, Wiley CA, Hansen LA. Lobar atrophy with dense core (brainstem type) Lewy bodies in a patient with dementia. Acta Neuropathol 80:453–8, 1990. [PMID: 2173332]
16. Ueda K, **Masliah** E, Saitoh T, Bakalis SL, Scoble H, Kosik KS. Alz–50 recognizes a phosphorylated epitope of tau protein. J Neurosci 10:3295–304, 1990. [PMID: 1698946]
17. **Masliah** E, Iimoto DS, Saitoh T, Hansen LA, Terry RD. Increased immunoreactivity of brain spectrin in Alzheimer disease: a marker of synapse loss? Brain Res 531:36–44, 1990. [PMID: 2289136]
18. **Masliah** E, Terry RD, Mallory M, Alford M, and Hansen LA. Diffuse plaques do not accentuate synapse loss in Alzheimer's disease. Am J Pathol 137:1293–7, 1990. [PMID: 2124413]

1991-1993

19. **Masliah** E, Terry RD, Alford M, DeTeresa R, Hansen LA. Cortical and subcortical patterns of synaptophysin–like immunoreactivity in Alzheimer disease. Am J Pathol 138:235–46, 1991. [PMID: 1899001]
20. **Masliah** E, Hansen LA, Quijada S, DeTeresa R, Alford M, Kauss J, Terry R. Late onset dementia with argyrophilic grains and subcortical tangles or atypical progressive supranuclear palsy? Ann Neurol 29:389-96, 1991. [PMID: 1929210]
21. Shapiro I P, **Masliah** E, Saitoh T. Altered protein tyrosine phosphorylation in Alzheimer's disease. J Neurochem 56:1152-62, 1991. [PMID: 1705956]
22. **Masliah** E, Hansen L, Albright T, Mallory M, Terry RD. Immunoelectron microscopic study of synaptic pathology in Alzheimer disease. Acta Neuropathol 81:428-33, 1991. [PMID: 1903014]
23. **Masliah** E, Yoshida K, Shimohama S, Gage F H, Saitoh T. Differential expression of protein kinase C isozymes in rat primary glial cultures. Brain Res 549:106-11, 1991. [PMID: 1893244]
24. Cole GM, **Masliah** E, Shelton ER, Chan H, Terry RD, Saitoh T. Accumulation of amyloid precursor fragment in Alzheimer plaque. Neurobiol Aging 12: 85-91, 1991. [PMID: 1711161]
25. Wiley CA, **Masliah** E, Morey M, Lemere C, DeTeresa R, Grafe M, Hansen L, Terry RD. Neocortical damage during HIV infection. Ann Neurol 29:651-7, 1991. [PMID: 1909852]
26. Saitoh T, **Masliah** E, Jin L–W, Cole GM, Wieloch T, Shapiro P. Protein kinases and phosphorylation in neurological disorders and cell death. Lab Invest 64:596-616, 1991. [PMID: 2030574]
27. **Masliah** E, Mallory M, Hansen L, Alford M, Albright T, DeTeresa R, Terry RD, Baudier J, Saitoh T. Patterns of aberrant sprouting in Alzheimer disease. Neuron 6:729-39, 1991. [PMID: 1827266]
28. Hansen LA, Natelson BH, Lemere C, Nieman W, DeTeresa R, Regan TJ, **Masliah** E, Terry RD. Alcohol-induced brain changes in dogs. Arch Neurol 48:939-42, 1991. [PMID: 1953418]

29. **Masliah** E, Fagan AM, Terry RD, DeTeresa R, Mallory M, Gage FH. Reactive synaptogenesis assessed by synaptophysin immunoreactivity is associated with GAP–43 in the dentate gyrus of the adult rat. Exp Neurol 113:131-42, 1991. [PMID: 1831150]

30. Armstrong TP, Hansen LA, Salmon DP, **Masliah** E, Pay M, Kunin JM, Katzman R. Rapidly progressive dementia in a patient with the Lewy body variant of Alzheimer's disease. Neurology 41:1178-80, 1991. [PMID: 1714056]

31. **Masliah** E, Cole GM, Hansen LA, Mallory M, Albright T, Terry RD, Saitoh T. Protein kinase C alteration is an early biochemical marker in Alzheimer's disease. J Neurosci 11:2759-67, 1991. [PMID: 1880547]

32. Terry RD, **Masliah** E, Salmon D, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R. Physical basis of cognitive alterations in Alzheimer disease: Synapse loss is the major correlate of cognitive impairment. Ann Neurol 30:572-80, 1991. [PMID: 1789684]

33. **Masliah** E, Hansen L, Mallory M, Albright T, Terry RD. Abnormal brain spectrin immunoreactivity in sprouting neurons in Alzheimer disease. Neurosci Lett 129:1-5, 1991. [PMID: 1922957]

34. Buzsaki G, **Masliah** E, Chen LS, Horvath Z, Terry RD, Gage FH. Hippocampal grafts into the intact brain induce epileptic patterns. Brain Res 554:30-7, 1991. [PMID: 1933310]

35. Hansen LA, **Masliah** E, Quijada–Fawcett S, Rexin D. Entorhinal neurofibrillary tangles in Alzheimer disease with Lewy bodies. Neurosci Lett 129:269-72, 1991. [PMID: 1745407]

36. **Masliah** E, Mallory M, Hansen L, Alford M, Albright T, Terry RD, Shapiro P, Sundsmo M, Saitoh T. Immunoreactivity of CD45, a protein tyrosine phosphatase, in Alzheimer's disease. Acta Neuropathol 83:12-20, 1991. [PMID: 1838850]

37. **Masliah** E, Ge N, Morey M, DeTeresa R, Terry RD, Wiley CA. Cortical dendritic pathology in human immunodeficiency virus encephalitis. Lab Invest 66:285-91, 1992. [PMID: 1538584]

38. Baum L, **Masliah** E, Iimoto DS, Hansen L, Halliday WC, Saitoh T. Casein kinase II is associated with neurofibrillary tangles but is not an intrinsic component of paired helical filaments. Brain Res 573:126-32, 1992. [PMID: 1576530]

39. **Masliah** E, Iimoto DS, Mallory M, Albright T, Hansen L, Saitoh T. Casein kinase alteration precedes tau accumulation in tangle formation. Am J Pathol 140:263-8, 1992. [PMID: 1739121]

40. **Masliah** E, Mallory M, Hansen L, Alford M, DeTeresa R, Terry RD, Baudier J, Saitoh T. Localization of amyloid precursor protein in GAP43-immunoreactive aberrant sprouting neurites in Alzheimer's disease. Brain Res 574:312-6, 1992. [PMID: 1386275]

41. **Masliah** E, Ellisman M, Carragher B, Mallory M, Young S, Hansen L, DeTeresa R, Terry RD. Three-dimensional analysis of the relationship between synaptic pathology and neuropil threads in Alzheimer disease. J Neuropathol Exp Neurol 51:404-14, 1992. [PMID: 1619440]

42. **Masliah** E, Achim CL, Ge N, DeTeresa R, Terry RD, Wiley CA. Spectrum of HIV associated neocortical damage. Ann Neurol 32:321-9, 1992. [PMID: 1416802]

43. Hessler D, Young SJ, Carragher BO, Martone M, Lamount S, Whittaker M, Milligan RA, **Masliah** E, Hinshaw JE, Ellisman M. Programs for visualization in three-dimensional microscopy. NeuroImage 1:55-67, 1992. [PMID: 9343557]

44. **Masliah** E, Mallory M, Ge N, Saitoh T. Amyloid precursor protein is localized in growing neurites of neonatal rat brain. Brain Res 593:323-8, 1992. [PMID: 1450940]

45. Connor DJ, Thal LJ, Mandel RJ, Langlais PL, **Masliah** E. Independent effects of age and nucleus basalis magnocellularis lesion: maze learning, cortical neurochemistry, and morphometry. Beh Neurosci 106:776-88, 1992. [PMID: 1280146]

46. **Masliah** E, Ge N, Achim C, Hansen LA, Wiley CA. Selective neuronal vulnerability in HIV encephalitis. J Neuropathol Exp Neurol 51:585-93, 1992. [PMID: 1484289]

47. **Masliah** E, Mallory M, Ge N, Saitoh T. Amyloid precursor protein is localized in growing neurites of neonatal rat brain. Brain Res 593:323-8, 1992. [PMID: 1450940]

48. Buzaki G, Hsu M, Horvath Z, Horsburgh K, Sundsmo M, **Masliah** E, Saitoh T. Kindling-induced changes of protein kinase C levels in hippocampus and neocortex. Epilepsy Res Suppl 9:279-83, 1992. [PMID: 1337442]

49. Saitoh T, **Masliah** E, Baum L, Sundsmo M, Flanagan L, Vikramkumar R, Kay M. Degradation of proteins in the membrane-cytoskeletal complex in Alzheimer's disease. Might amyloidogenic APP processing be just the tip of the iceberg? Ann NY Acad Sci 674:180-92, 1992. [PMID: 1288363]

50. **Masliah** E, Mallory M, Hansen L, DeTeresa R, Terry RD. Quantitative synaptic alterations in the human neocortex during normal aging. Neurology 43:192-7, 1993. [PMID: 8423884]

51. **Masliah** E, Mallory M, Hansen L, Alford M, DeTeresa R, Terry RD. An antibody against phosphorylated neurofilaments identifies a subset of damaged associated axons in Alzheimer's disease. Am J Pathol 142:871-82, 1993. [PMID: 8456946]

52. **Masliah** E, Terry RD. The role of synaptic proteins in the pathogenesis of disorders of the central nervous system. Brain Pathol 3:77-85, 1993. [PMID: 8269086]

53. Lev-Ram V, Valsamis M, **Masliah** E, Levine S, Godfrey HP. A novel non-ataxic guinea pig strain with cerebrocortical and cerebellar abnormalities. Brain Res 606:325-31, 1993. [PMID: 8490725]

54. **Masliah** E, Mallory M, DeTeresa R, Alford M, Hansen L. Differing patterns of aberrant neuronal sprouting in Alzheimer disease with and without Lewy bodies. Brain Res 617:258-66, 1993. [PMID: 8402154]

55. **Masliah** E, Miller A, Terry RD. The synaptic organization of the neocortex in Alzheimer's disease. Med Hypoth 41:334-40, 1993. [PMID: 8289698]

56. Reyes E, Gamboa A, **Masliah** E. Atypical Lewy body disease with neuritic abnormalities. Clin Neuropathol 12:330-4, 1993. [PMID: 8287626]

57. Langlais PJ, Thal L, Hansen L, Galasko D, Alford M, **Masliah** E. Neurotransmitters in basal ganglia and cortex of Alzheimer's disease with and without Lewy bodies. Neurology 43:1927-34, 1993. [PMID: 8105420]

58. **Masliah** E, Mallory M, Alford M, Saitoh T. Immunoreactivity of the nuclear antigen p105 is associated with tangle and plaque formation in Alzheimer's disease. Lab Invest 69:562-9, 1993. [PMID: 8246448]

59. **Masliah** E, Mallory M, Ge N, Godson C, Saitoh T. Phorbol ester-induced neuritic alterations in the rat neocortex. Structural and immunocytochemical studies. Mol Chem Neuropath 20:125-45, 1993. [PMID: 8297418]

60. **Masliah** E, Mallory M, Deerinck T, DeTeresa R, Lamont S, Miller A, Terry RD, Carragher B, Ellisman M. Re-evaluation of the organization of neuritic plaques in Alzheimer's disease. J Neuropathol Exp Neurol 52:619-32, 1993. [PMID: 8229081]

61. Ueda K, Fukushima H, **Masliah** E, Xia Y, Iwai A, Otero D, Kondo J, Ihara Y, Saitoh T. Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. Proc Natl Acad Sci USA 90:11282-6, 1993. [PMID: 8248242]

62. Hansen LA, **Masliah** E, Galasko D, Terry RD. Plaque only Alzheimer disease is usually the Lewy body variant, and vice versa. J Neuropathol Exp Neurol 52:648-54, 1993. [PMID: 8229084]

63. Campbell I, Abraham CR, **Masliah** E, Kemper Ph, Inglis JD, Oldstone MBA, Mucke L. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin-6. Proc Natl Acad Sci USA 90:10061-5, 1993. [PMID: 7694279]

64. Nagra RM, **Masliah** E, Wiley C. Synaptic and dendritic pathology in murine retroviral encephalitis. Exp Neurol 124:283-8, 1993. [PMID: 8287927]

65. Saitoh T, Horsburg K, **Masliah** E. Hyperactivation of signal transduction systems in Alzheimer's disease. Ann NY Acad Sci 695:34-41, 1993. [PMID: 8239309]

66. **Masliah** E, Terry R. The role of synaptic pathology in the mechanisms of dementia in Alzheimer's disease. Clin Neurosci 1:192-8, 1993.

67. Toggas SM, **Masliah** E, Rockenstein EM, Rall GF, Abraham CR, Mucke L. Central nervous system damage produced by expression of the HIV-1 coat protein gp120 in transgenic mice. Nature 367:188-93, 1994. [PMID: 8114918]

68. Alford MF, **Masliah** E, Hansen L, Terry RD. A simple dot-immunobinding assay for quantification of synaptophysin-like immunoreactivity in human brain. J Histochem Cytochem 42:283-7, 1994. [PMID: 8288869]

69. **Masliah** E, Honer WG, Mallory M, Voigt M, Kushner P, Terry R. Topographical distribution of synaptic-associated proteins in the neuritic plaques of Alzheimer disease hippocampus. Acta Neuropathol 87:135-42, 1994. [PMID: 8171963]

70. Roch J-M, **Masliah** E, Roch-Levecq AC, Sundsmo M, Otero D, Veinbergs I, Saitoh T. Increase of synaptic density and memory retention by a peptide representing the trophic domain of the amyloid ß/A4. Proc Natl Acad Sci USA 91:7650-4, 1994. [PMID: 8052602]

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72. Samuel W, Terry RD, DeTeresa R, Butters N, **Masliah** E. Clinical correlates of cortical and nucleus basalis pathology in Alzheimer dementia. Arch Neurol 51:772-8, 1994. [PMID: 8042925]

73. Bowes MP, **Masliah** E, Chen B-L, Otero D, Zivin J, Saitoh T. Reduction of neurological damage by a trophic peptide segment of the amyloid ß/A4 protein (APP). Exp Neurol 129:1-8, 1994. [PMID: 7925833]

74. Mucke L, **Masliah** E, Johnson WB, Ruppe M, Alford M, Rockenstein EM, Forss-Petter S, Pietropaolo M, Mallory M, Abraham C. Synaptotrophic effects of human amyloid beta protein precursors in the cortex of transgenic mice. Brain Res 666:151-67, 1994. [PMID: 7882025]

75. Samuel W, **Masliah** E, Terry R. Hippocampal connectivity and Alzheimer's dementia: effects of pathology in a two-component model. Neurology 44:2081-8, 1994. [PMID: 7969963]

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Passive immunotherapy against Aβ in aged APP-transgenic mice reverses cognitive deficits and depletes parenchymal amyloid deposits in spite of increased vascular amyloid and microhemorrhage

Donna M Wilcock[1], Amyn Rojiani[2], Arnon Rosenthal[3], Sangeetha Subbarao[3], Melissa J Freeman[1], Marcia N Gordon[1] and Dave Morgan*[1]

Address: [1]Alzheimer's Research Laboratory, University of South Florida, Department of Pharmacology, 12901 Bruce B Downs Blvd, Tampa, Florida 33612, USA, [2]Alzheimer's Research Laboratory, University of South Florida, Department of Interdisciplinary Oncology, 12901 Bruce B Downs Blvd, Tampa, Florida 33612, USA and [3]Rinat Neuroscience Corp., 3155 Porter Drive, Palo Alto, California 94304, USA

Email: Donna M Wilcock - dwilcock@hsc.usf.edu; Amyn Rojiani - arojiani@hsc.usf.edu; Arnon Rosenthal - ar@rinatneuro.com; Sangeetha Subbarao - Sangeetha@rinatneuro.com; Melissa J Freeman - mfreema1@hsc.usf.edu; Marcia N Gordon - mgordon@hsc.usf.edu; Dave Morgan* - dmorgan@hsc.usf.edu

* Corresponding author

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Abstract

Background: Anti-Aβ immunotherapy in transgenic mice reduces both diffuse and compact amyloid deposits, improves memory function and clears early-stage phospho-tau aggregates. As most Alzheimer disease cases occur well past midlife, the current study examined adoptive transfer of anti-Aβ antibodies to 19- and 23-month old APP-transgenic mice.

Methods: We investigated the effects of weekly anti-Aβ antibody treatment on radial-arm water-maze performance, parenchymal and vascular amyloid loads, and the presence of microhemorrhage in the brain. 19-month-old mice were treated for 1, 2 or 3 months while 23-month-old mice were treated for 5 months. Only the 23-month-old mice were subject to radial-arm water-maze testing.

Results: After 3 months of weekly injections, this passive immunization protocol completely reversed learning and memory deficits in these mice, a benefit that was undiminished after 5 months of treatment. Dramatic reductions of diffuse Aβ immunostaining and parenchymal Congophilic amyloid deposits were observed after five months, indicating that even well-established amyloid deposits are susceptible to immunotherapy. However, cerebral amyloid angiopathy increased substantially with immunotherapy, and some deposits were associated with microhemorrhage. Reanalysis of results collected from an earlier time-course study demonstrated that these increases in vascular deposits were dependent on the duration of immunotherapy.

Conclusions: The cognitive benefits of passive immunotherapy persist in spite of the presence of vascular amyloid and small hemorrhages. These data suggest that clinical trials evaluating such treatments will require precautions to minimize potential adverse events associated with microhemorrhage.

Background

Alzheimer's disease is characterized not only by the presence of parenchymal amyloid deposits and intracellular tangles but also by the presence of amyloid deposits in the vasculature, a condition referred to as cerebral amyloid angiopathy (CAA). The CAA observed in both Alzheimer's disease patients [1] and some of the transgenic mouse models [2] is primarily composed of the shorter form of amyloid beta (Aβ), $A\beta_{1-40}$, while the majority of amyloid deposits in the parenchyma are composed of $A\beta_{1-42}$, although the compact amyloid deposits also contain $A\beta_{1-40}$.

Anti-Aβ immunotherapy has been considered as a potential treatment for Alzheimer's disease for some time [3,4]. Active immunization with a vaccine including $A\beta_{1-42}$ fibrils progressed to human clinical trials where its administration was suspended due to meningoencephalitits in a subset of patients [5]. To date there have been pathology reports on two patients who participated in the trial and subsequently died [6,7]. Both reports note that while the numbers of parenchymal amyloid deposits appeared lower than expected in these cases, the CAA in these patients did not appear outside the normal range for Alzheimer's disease. In addition, one report mentioned multiple cortical hemorrhages and the presence of hemosiderin around the CAA vessels [7].

Given the adverse reactions to the active immunization, the irreversibility of such procedures and the variable antibody response to vaccines in older individuals [8], passive immunization against the Aβ peptide emerged as an alternative immunotherapeutic strategy. Studies in young and middle aged APP-transgenic mice have reported significant amyloid reductions with passive immunization [9-11]. Such treatments also demonstrate rapid improvements of memory function in APP-transgenic mice, sometimes without detectable reductions in amyloid [12-14]. Most recently, intracranial administration of anti-Aβ antibodies has been shown to not only remove Aβ but also clear, early-stage, hyperphosphorylated-tau aggregates [15]. Importantly, in the only prior study evaluating adoptive antibody transfer in older APP-transgenic mice, Pfeifer *et al.* [16] reported a doubling of cerebral microhemorrhages associated with significant reductions in amyloid burden after administration of an N-terminal specific anti-Aβ antibody.

Materials and Methods

Experiment design

Mice derived from APP Tg2576 mice were obtained from our breeding program at University of South Florida started in 1996 [17]. For the 5-month treatment study, 13 APP-transgenic mice, aged 23 months, were assigned to one of two groups. The first group received weekly intraperitoneal anti-Aβ antibody injections (antibody 2286; mouse-monoclonal anti-human $A\beta_{28-40}$ IgG1; Rinat Neurosciences, Palo Alto, CA) for a period of 5 months (n = 6). The second group received weekly intraperitoneal anti-AMN antibody (2906; mouse-monoclonal anti-*Drosophila* amnesiac protein IgG1; Rinat Neurosciences, Palo Alto, CA) injections for a period of 5 months (n = 7). Seven nontransgenic mice were also assigned to one of two groups. The first group received weekly intraperitoneal anti-Aβ antibody injections for a period of 5 months (n = 4). The second group received weekly intraperitoneal anti-AMN antibody injections for a period of 5 months (n = 3).

For the time course study of 1-, 2- or 3-month treatment, 22 APP-transgenic mice aged 19 months were assigned to one of four experimental groups, as described previously [14]. The first three groups received weekly intraperitoneal anti-Aβ antibody injections for 3 months, 2 months or 1 month, ending when all mice were 22 months of age. The fourth group received weekly intraperitoneal anti-AMN antibody injections for 3 months.

Behavioral analysis

Following 3 and 5 months of treatment, the mice from the 5-month study were subjected to a two-day radial-arm water-maze paradigm. The apparatus was a 6-arm maze as described previously [18]. On day one, 15 trials were run in three blocks of 5. A cohort of 4 mice were run sequentially for each block (i.e., each of 4 mice get trial one, then the same mice get trial two, etc.). After each 5-trial block, a second cohort of mice was run permitting an extended rest period before mice were exposed to the second block of 5 trials. The goal arm was different for each mouse in a cohort to minimize odor cues. The start arm was varied for each trial, with the goal arm remaining constant for a given individual for both days. For the first 11 trials, the platform was alternately visible then hidden (hidden for the last 4 trials). On day two, the mice were run in exactly the same manner as day one except that the platform was hidden forall trials. The number of errors (incorrect arm entries) was measured in a one-minute time frame. As standard practice, mice failing to make an arm choice in 20 seconds are assigned one error, but no mice in this study had to be assigned an error in this manner. The same individual administered the antibody treatments and placed mice in the radial-arm water maze. Due to the numbers of mice in the study the researcher was unaware of treatment group identity of each mouse. Also, the dependent measures in the radial-arm water-maze task are quantitative, not evaluative, so the potential for tester bias is reduced. In order to minimize the influence of individual trial variability, each mouse's errors for 3 consecutive trials were averaged producing 5 data points for each day, which were analyzed statistically by ANOVA using StatView (SAS Institute Inc., NC).

Tissue preparation and histology

On the day of sacrifice mice were weighed, overdosed with 100 mg/kg Nembutal (Abbott laboratories, North Chicago, IL), and then intracardially perfused with 25 mL of 0.9% sodium chloride. Brains were rapidly removed, and the left half of the brain was immersion fixed for 24 h in freshly prepared 4% paraformaldehyde in 100 mM KPO_4 (pH 7.2) for histopathology. The hemi-brains were then incubated for 24 h in 10%, 20% and 30% sucrose sequentially for cyroprotection. Horizontal sections of 25 μ thickness were collected using a sliding microtome and stored at 4°C in Dulbecco's phosphate-buffered saline with sodium azide (pH 7.2) to prevent microbial growth. A series of 8 equally spaced tissue sections 600 μ apart were randomly selected spanning the entire brain and stained using free-floating immunohistochemistry for total Aβ (rabbit polyclonal anti-pan Aβ; Biosource, Camarillo, CA, 1:10,000) as previously described [2,14]. A second series of tissue sections 600 μm apart were stained using 0.2% Congo red in NaCl-saturated 80% ethanol. Another set of sections were also mounted and stained for hemosiderin using 2% potassium ferrocyanide in 2% hydrochloric acid for 15 min, followed by a counterstain in a 1% neutral red solution for 10 min. Quantification of Congo red staining and Aβ immunohistochemistry was performed using the Image-Pro Plus (Media Cybernetics, Silver Spring, MD) to analyze the percent area occupied by positive stain. One region of the frontal cortex and three regions of the hippocampus were analyzed (to ensure that there was no regional bias in the hippocampal values). The initial analysis of Congo red was performed to give a total value. A second analysis was performed after manually editing out all of the parenchymal amyloid deposits to yield a percent area restricted to vascular Congo red staining. To estimate the parenchymal area of Congo red, we subtracted the vascular amyloid values from the total percentage. For the hemosiderin stain the numbers of Prussian blue-positive sites were counted on all sections and the average number of sites per section calculated. Looking at the sections at a low magnification we were able to observe a qualitative differences between animals; however, the percent area was so low that many fields contained no positive stain. Eight equally spaced sections were examined and the number of positive profiles was determined and averaged to a per-section value. To assess possible treatment-related differences, the values for each treatment group were analyzed by one-way ANOVA followed by Fisher's LSD means comparisons.

Results

Reversal of cognitive deficits by passive amyloid immunotherapy

The radial-arm water-maze task detects spatial learning and memory deficits in transgenic mouse models [18,19]. We treated 23-month-old mice for 5 months with anti-Aβ antibody 2286 or control antibody 2906 (against a *Drosophila*-specific protein) and tested them for spatial navigation learning in a two-day version of the radial-arm water maze after 3 months of treatment and, using a new platform location, again after 5 months of treatment. At both testing times we found that APP-transgenic mice treated with the control antibody failed to learn platform location over two days of testing and were significantly impaired compared to the nontransgenic mice treated with either antibody (Fig. 1). However, APP-transgenic mice administered the anti-Aβ antibodies demonstrated a complete reversal of the impairment observed in the control-treated APP-transgenic mice, ending day two with a mean performance near 0.5 errors per trial (Fig. 1). Although learning at the later time point, when the mice were 28 months of age, may have been slightly slower for all groups, there was no impairment of the anti-Aβ antibody-treated APP.

Passive amyloid immunotherapy clears parenchymal Aβ deposits, but increases vascular amyloid

In a prior experiment examining the effects of passive anti-Aβ immunotherapy for 1, 2 or 3 months in APP-transgenic mice killed at 21 months of age [14], we found a time-dependent reduction of both Aβ immunostaining of diffuse and fibrillar deposits and Congo-red staining of fibrillar amyloid deposits. In the current study we found a similar reduction in both Aβ immunostaining (Table 1) and total Congo-red staining (Fig. 2A, left panel; $p < 0.001$ frontal cortex and $p < 0.01$ hippocampus) after 5 months of immunotherapy. We noted that the bulk of what remained was vascular amyloid. We then separately analyzed vascular and parenchymal deposits which revealed a near 90% reduction in parenchymal deposits ($p < 0.001$) but a 3–4 fold elevation of vascular Congo-red staining ($p < 0.0001$; Fig. 2A, center and right panels, respectively). We also separately analyzed vascular and parenchymal Congo-red staining on mice from our earlier study [14], treated passively for 1, 2 or 3 months with anti-Aβ or control antibody, and found a similar result. There was a graded reduction in overall Congo-red staining nearing 75% as duration of antibody exposure increased (as reported previously; Fig. 2B). However, when separated into vascular Congo-red deposits and parenchymal deposits, there was an antibody-exposure-time-dependent increase in vascular deposition in both hippocampus and frontal cortex (Fig. 2C; $p < 0.05$ frontal cortex and hippocampus) and a corresponding nearly 90% decrease in parenchymal deposits (Fig. 2D; $p < 0.001$ in frontal cortex and hippocampus).

These differences were readily observed examining micrographs of sections from these mice. Mice treated with control antibodies revealed occasional cortical vascular

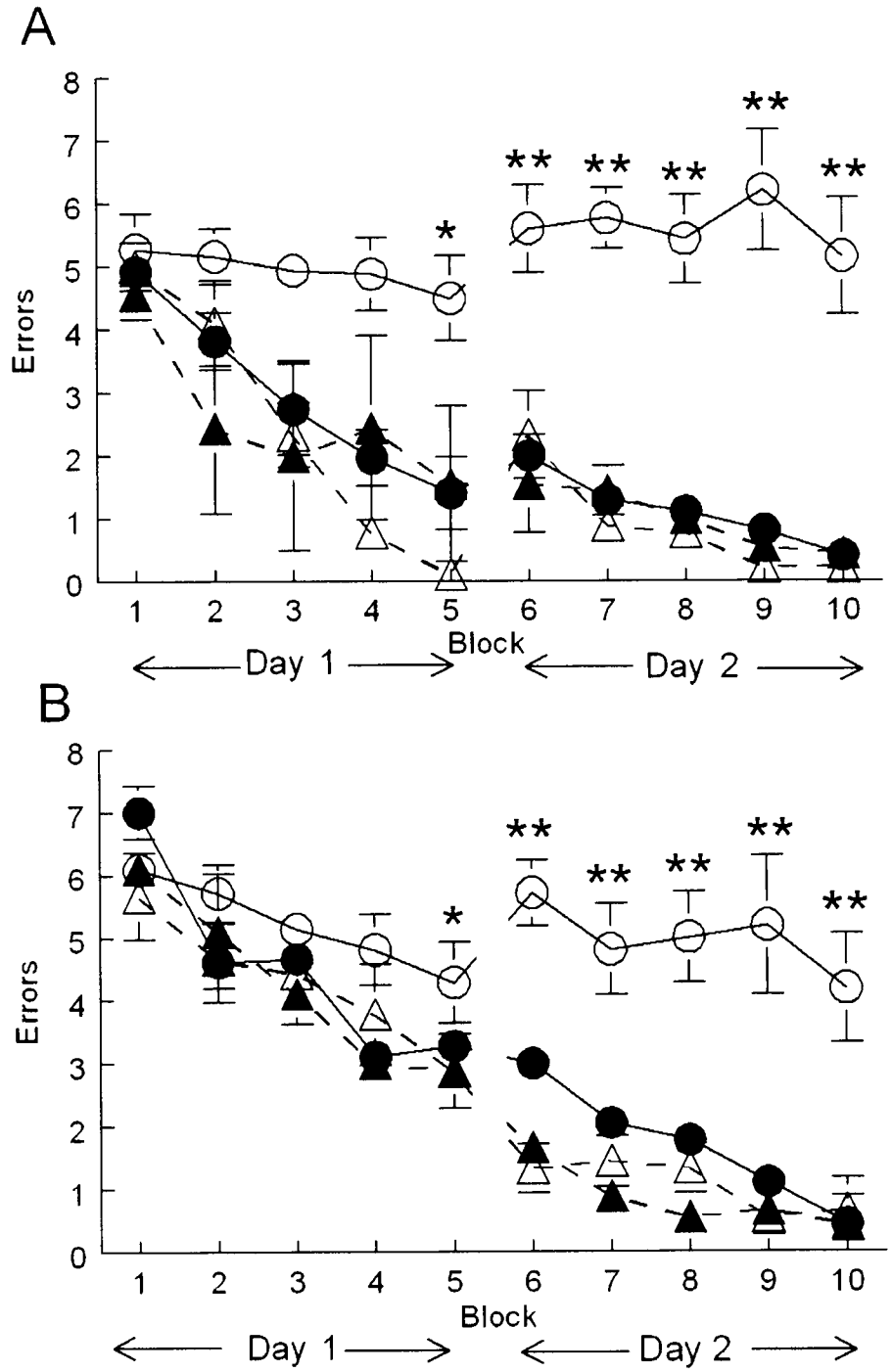

Figure 1
Spatial learning deficits in APP-transgenic mice were reversed following 3 and 5 months of immunization. Mice were tested in a two-day version of the radial-arm water maze. Solid lines represent APP-transgenic mice while dashed lines represent non-transgenic mice. Open symbols indicate anti-AMN, control-antibody treatment (○: APP-transgenic, control antibody; △: non-transgenic, control antibody) while closed symbols indicate anti-Aβ antibody treatment (●: APP-transgenic, Aβ antibody; ▲: nontransgenic, Aβ antibody). Panel A shows mean number of errors made over the two-day trial period following 3 months of immunization. Each data point is the average of 3 trials. Panel B shows the mean number of errors made over the 2-day trial period following 5 months of immunization. For both graphs * indicates $p < 0.05$, ** indicates $p < 0.001$ when the APP-transgenic mice receiving control antibody are compared with the remaining groups.

Table 1: Total Aβ load is significantly reduced following 5 months of anti-Aβ antibody treatment. Percent area occupied by positive immunohistochemical stain for Aβ is shown ± standard error of the mean for both the frontal cortex and hippocampus. Also shown is the percent reduction of Aβ observed following anti-Aβ antibody treatment

Region	% area positive for Aβ: control treated	% area positive for Aβ: anti-Aβ treated	% reduction following anti-Aβ antibody treatment
Frontal Cortex	34.855 ± 2.265	9.681 ± 0.754	72
Hippocampus	23.994 ± 0.985	8.212 ± 0.596	66

amyloid deposits (22 months, Fig. 3A, 28 months, Fig. 3C), while mice administered anti-Aβ antibodies had increased amounts of vascular amyloid staining (3-month treatment, Fig 3B; 5-month treatment, Fig 3D). Those vessels containing amyloid following treatment with anti-Aβ antibody also exhibited apparent increases in microglial activation as measured by CD45 expression (Fig. 3F) compared to mice treated with control antibody (Fig. 3E). Unfortunately, the shifting numbers and sizes of vascular and parenchymal deposits caused by the antibody therapy greatly complicated measurement of microglial activation per vascular deposit area so that this apparent increase in staining intensity could not be quantified accurately.

Passive amyloid immunotherapy causes increased microhemorrhage

We used the Prussian blue histological stain to label hemosiderin, a ferric oxide material produced in the breakdown of hemoglobin. Extravenous blood in the brain leads to microglial phagocytosis of the erythrocytes and breakdown of the hemoglobin within them. These ferric oxide-containing microglia are thus markers of past hemorrhage. In untreated, aged APP-transgenic mice we observed very few profiles positive for Prussian-blue staining in the frontal cortex (section counterstained with neutral red; Fig. 4A). However, following anti-Aβ antibody treatment for 5 months we observed an increase in the number of Prussian-blue profiles in the frontal cortex, which were readily detectable at a low magnification in the microscope (Fig. 4B). In the absence of anti-Aβ treatment, or even when treated with antibody for one month, most vessels did not stain with Prussian blue, and could be identified only using the red counterstain (Fig. 4C). However, even with 3 months of anti-Aβ antibody treatment we observed frequent vessels with associated Prussian-blue staining (Fig 4D). Using adjacent sections stained for Congo red, we confirmed that all vessels showing microhemorrhage contained amyloid (Figs. 4E and 4F; we were unable to double-label Prussian blue-stained sections with either Congo red or thioflavine-S). However, only a minority of vessels containing amyloid demonstrated hemorrhage.

When we counted the number of Prussian blue-positive profiles in those animals receiving control antibody there was an average of one profile per every two sections (Fig. 5) and this number remained the same in both control groups (aged 22 or 28 months). Following treatment with anti-Aβ antibody for a period of two months we observed a striking increase in Prussian-blue staining, approximately five times that observed in either the control group or the mice immunized for one month (Fig. 5, $p < 0.001$). Following this initial increase in Prussian-blue staining, we observed a linear increase in staining associated with increasing duration of anti-Aβ antibody treatment (Fig 5). Five months of anti-Aβ antibody treatment demonstrated a six-fold increase in Prussian-blue staining when compared the control groups (Fig. 5).

Discussion

Earlier studies with vaccines against the Aβ peptide demonstrated protection from the learning and memory deficits associated with amyloid accumulation in APP-transgenic mice [14,19]. Passive immunization protocols with anti-Aβ antibodies also produced cognitive benefits, in some cases even in the absence of significant reduction in amyloid burden [12,13]. Our recent work found that 3 months of anti-Aβ treatment of 18-month-old APP-transgenic mice improved spontaneous alternation performance on the Y-maze [14]. In the present work we confirmed that passive anti-amyloid immunotherapy can reverse spatial learning deficits in APP-transgenic mice and that this benefit of immunotherapy is retained, even in aged mice (26 and 28 months old at testing) with long-established amyloid pathology.

Additionally, we describe a more rapid means of testing spatial reference memory to reveal learning and memory deficits in APP-transgenic mice. This two-day version of the radial arm water maze included greater spacing of individual trials (mice spent time in their home cage after every trial), combined with less spacing of aggregate trials (fifteen trials per day rather than four or five) to facilitate learning of platform location in the nontransgenic mice, with a clear absence of learning in the age-matched transgenic mice.

A substantial reduction in total Congophilic amyloid deposits was observed in old APP-transgenic mice treated with anti-Aβ antibodies for 2 or more months. This

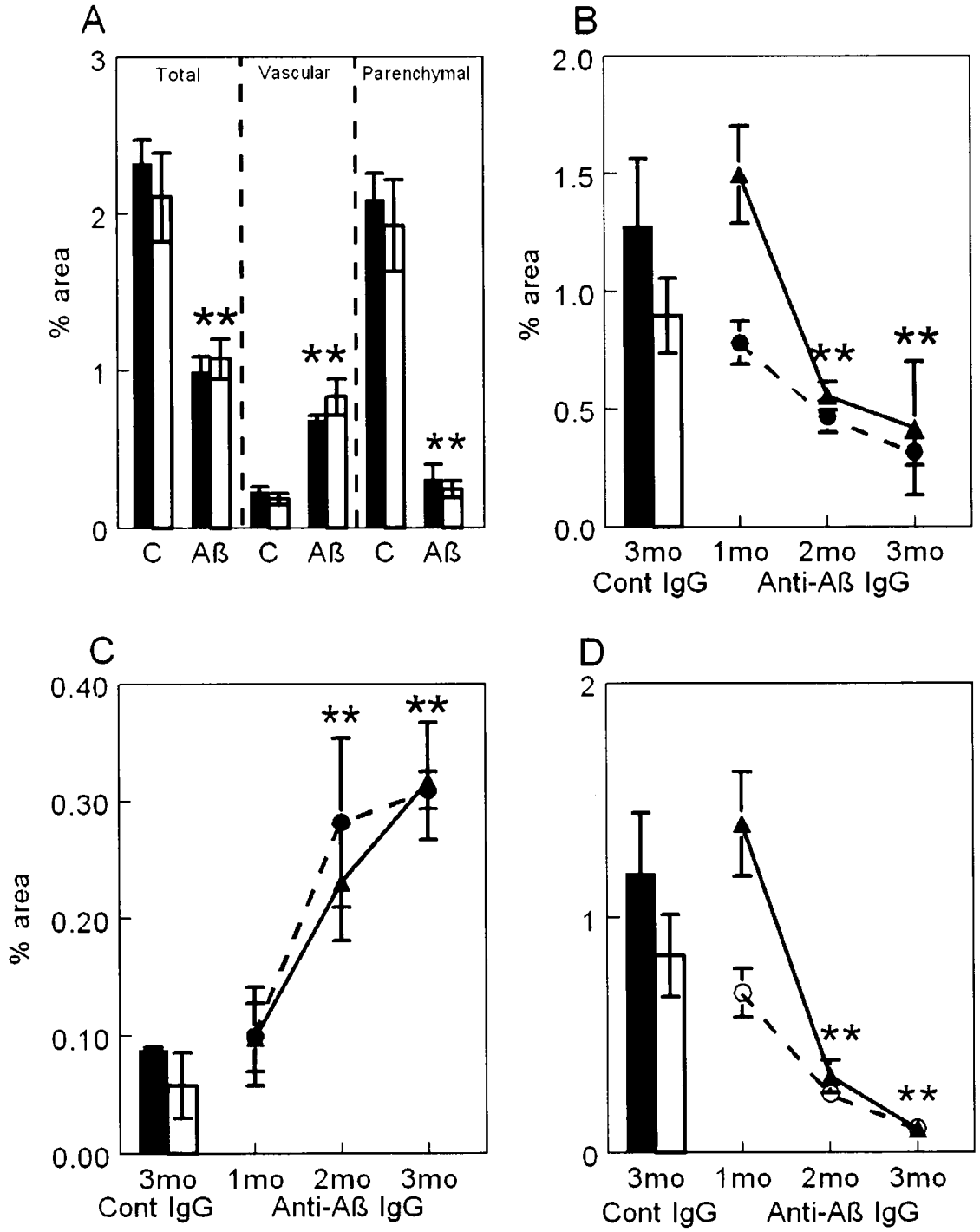

Figure 2

Passive immunization with anti-Aβ antibodies decreases total and parenchymal amyloid loads while increasing vascular amyloid in frontal cortex and hippocampus of APP-transgenic mice. Panel A shows total amyloid load measured with Congo red, vascular amyloid load and parenchymal amyloid load from APP-transgenic mice administered control IgG (C) or anti-Aβ IgG (Aβ) for a period of 5 months. Panels B-D show total amyloid load (Panel B), vascular amyloid load (Panel C) and parenchymal amyloid load (Panel D) from APP-transgenic mice administered control IgG for 3 months (Cont IgG) or anti-Aβ IgG for a period of 1, 2, or 3 months (Anti-Aβ IgG). For all panels, the solid bar and solid line represent values from the frontal cortex, while the open bar and dashed line represent values from the hippocampus. ** $p < 0.01$.

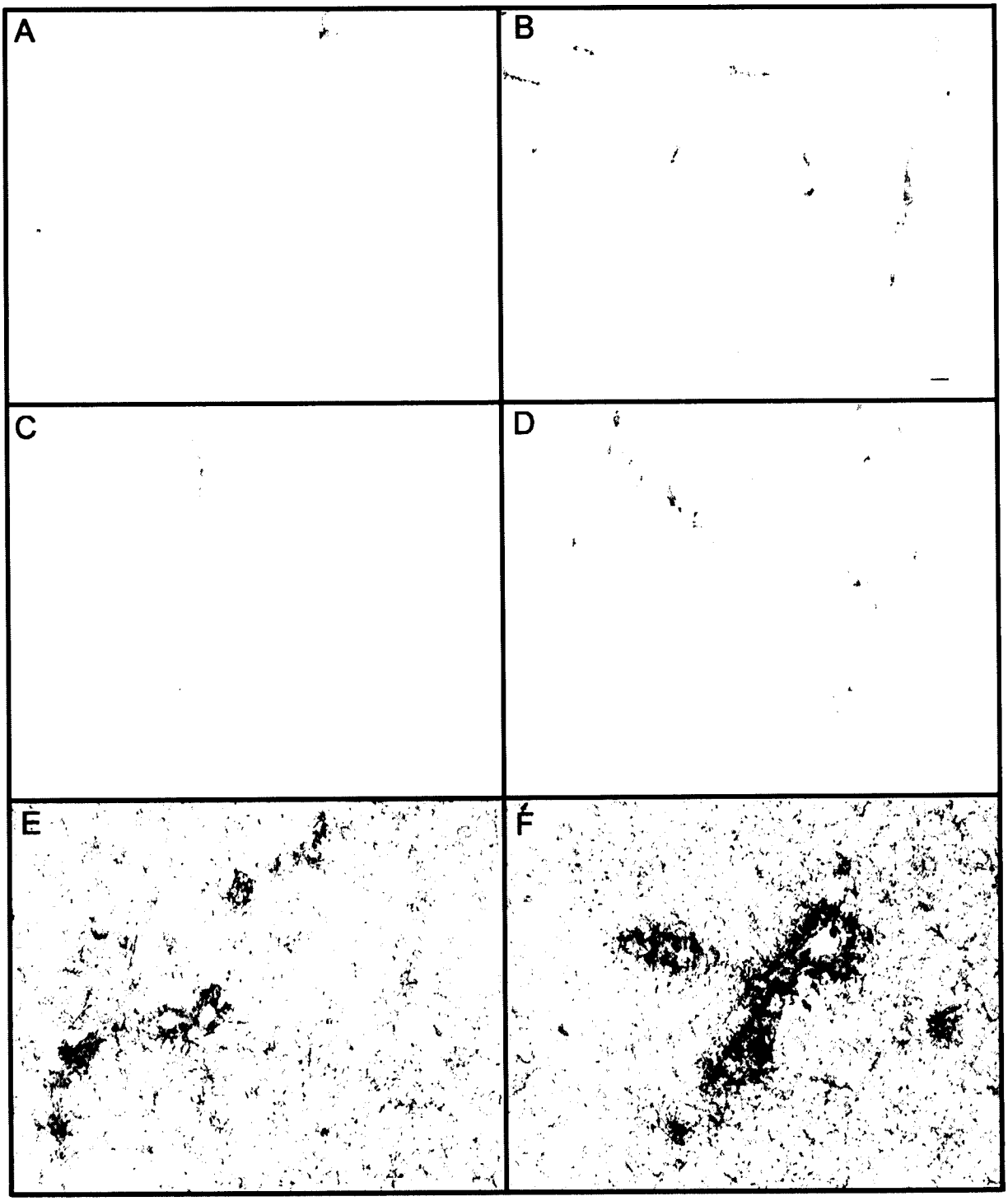

Figure 3
Increased Congo red staining of blood vessels following anti-Aβ antibody administration is associated with activated microglia. Panels A and B are from the frontal cortex of 22-month-old APP-transgenic mice immunized for 3 months with either control antibody (3A) or anti-Aβ antibody (3B). Panels C and D are from the frontal cortex of 28-month-old APP-transgenic mice immunized for 5 months with either control antibody (3C) or anti-Aβ antibody (3D). Panels E and F show a high-magnification image of CD45 immunohistochemistry (black) counterstained with Congo red (red) from 28-month-old APP-transgenic mice immunized for 5 months with either control antibody (Panel E) or anti-Aβ antibody (Panel F). Panels A-D, magnification = 100X. Scale bar in Panel B = 50 μ for panels A-D. Panels E-F, magnification = 200X. Scale bar in Panel E = 25 μm for panels E-F.

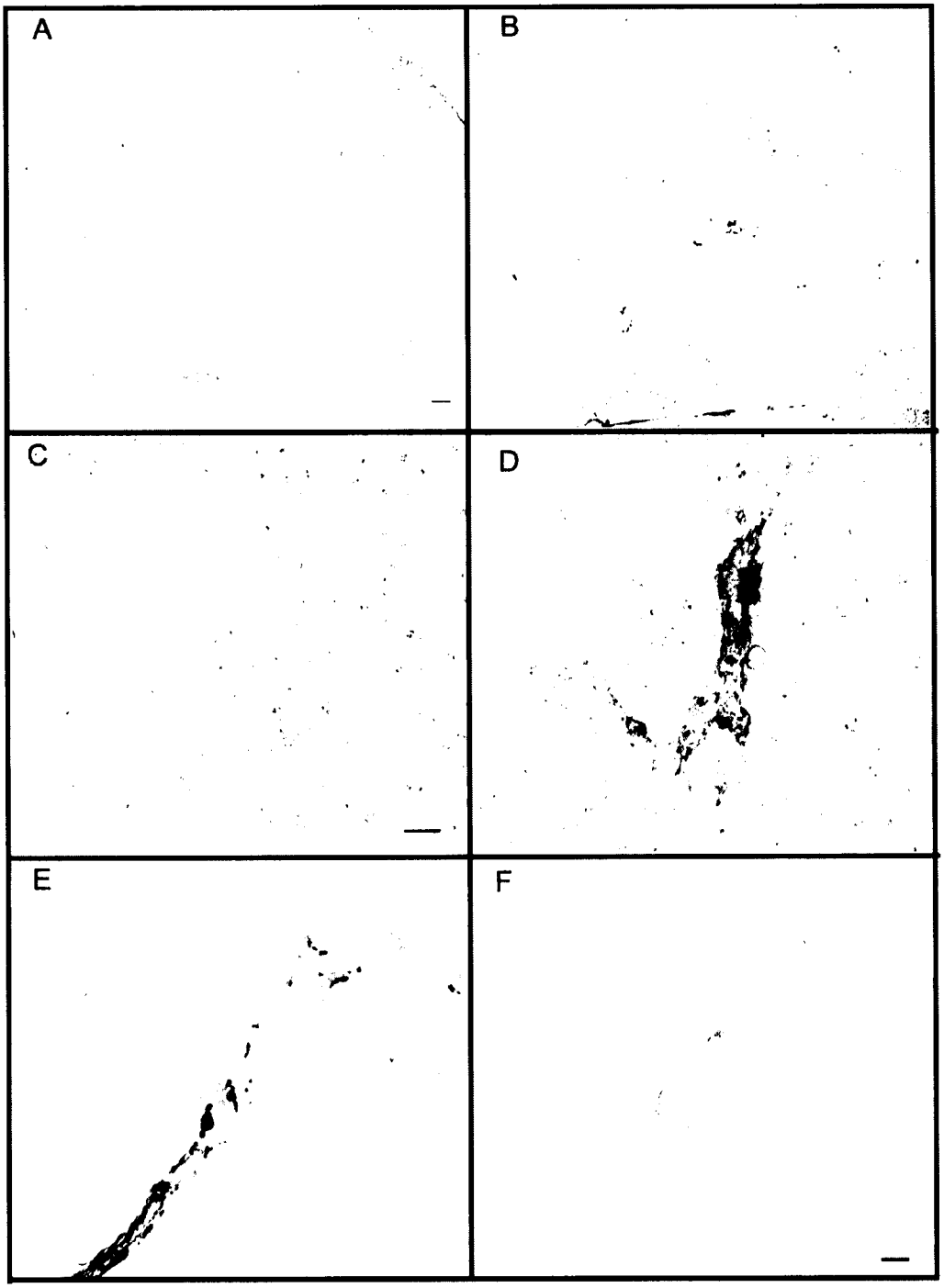

Figure 4
Microhemorrhage associated with CAA following systemic administration of anti-Aβ antibodies. Panels A and B are low magnification images of the frontal cortex of APP-transgenic mice receiving either control antibodies (Panel A) or anti-Aβ antibodies (Panel B) for a period of 5 months. Panels C and D show representative images of amyloid containing vessels stained for Prussian blue (blue), counterstained with neutral red (red), from APP-transgenic mice receiving either control antibodies (Panel C) or anti-Aβ antibodies (Panel D) for a period of 3 months. Panel E shows a blood vessel in the frontal cortex stained for Prussian blue (blue), counterstained with neutral red, from an APP transgenic mouse administered anti-Aβ antibodies for 5 months. Panel F shows the same blood vessel on an adjacent section stained for Congo red, indicating that the blood vessel does in fact contain amyloid. Scale bar panel A = 120 μm for panels A-B. Scale bar panel C = 25 μm for panels C-D. Scale bar in panel F = 25 μm for panels E-F.

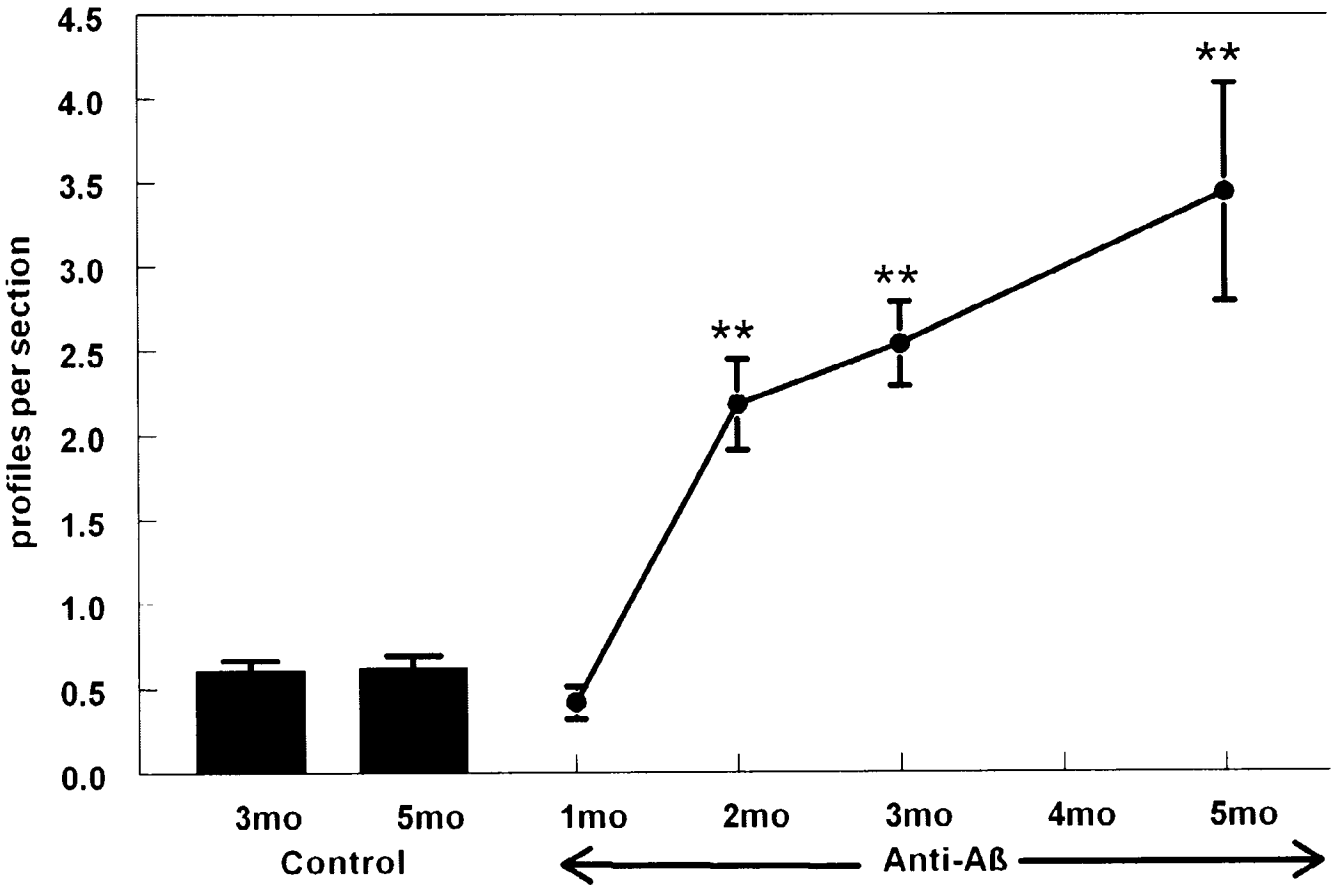

Figure 5
Number of Prussian blue-positive profiles increases with duration of anti-Aβ antibody exposure. The graph shows quantification of the average number of Prussian blue-positive profiles per section from mice administered control IgG for 3 or 5 months (Cont) or anti-Aβ IgG for 1, 2, 3 or 5 months (anti-Aβ). ** $p < 0.01$.

measurement of total Congo-red staining included both parenchymal and vascular amyloid staining. When we analyzed the sections for only vascular amyloid (CAA) we found that this measure was significantly increased following 2, 3 and 5 months of anti-Aβ antibody treatment. The remaining parenchymal amyloid load was almost completely eliminated with this antibody approach. Clearly, because total amyloid load was significantly reduced not all amyloid was shifted into the vessels; but, it appears that at least some of the Congophilic material was redistributed to the vasculature. At the present time the mechanism for this redistribution is unclear. However, one possibility is that the microglia associated with the antibody-opsonized amyloid, either by phagocytosis or surface binding, and transported the material to the vasculature, possibly in an attempt to expel it. We and others have shown evidence for microglial involvement in the removal of amyloid using both intracranial anti-Aβ antibody injections [11,21] and systemically administered anti-Aβ antibody treatment [14], as well as *ex vivo* studies [10,22]. Here we also report our impression that microglia surrounding CAA vessels in immunized mice expressed more CD45 than control transgenic mice. This increased expression could be due to either increased expression in the same number of microglial cells or an increased number of microglial cells in these animals. It is feasible that this microglial activation was simply in reaction to the presence of increased amyloid in the blood vessels. However, it is equally likely that microglia activated by the opsonized material migrated to the vessels for disposal of the amyloid.

Cerebral amyloid angiopathy (CAA) is defined as the deposition of congophilic material in meningeal and cerebral arteries and arterioles (capillaries and veins can also show CAA but less frequently), and it occurs to some extent in

nearly all Alzheimer's disease patients [23]. Severe CAA, affecting about 15% of cases, can be associated with both infarction and hemorrhagic injury [24,25]. It has also been shown that the severity of CAA can be directly linked to the severity of dementia in Alzheimer's disease patients [26].

In the current study we found a significantly increased number of microhemorrhages in the brain as detected by Prussian-blue staining, associated with the increase in CAA following passive immunization. Another transgenic mouse model of amyloid deposition, the APP23 mice, have been shown to deposit amyloid in both brain parenchyma and blood vessels and show a CAA associated increase in spontaneous cerebral hemorrhages [27]. Moreover, Pfeifer *et al.* [16] showed that these spontaneous hemorrhages were significantly increased following 5 months of passive immunization of 21-month-old APP23 mice using an anti-Aβ antibody with an N-terminal epitope, similar to those typically developed in active immunization with vaccines [4,28,29]. When young mice (6 months of age) were immunized following the same protocol, no hemorrhages were observed. More recently, DeMattos *et al.* [30] showed that passive immunization with an N-terminal antibody (3D6: directed against amino acids 1–5 of Aβ) of PDAPP transgenic mice also resulted in significantly increased microhemorrhage. They were unable to detect increased microhemorrhage with a mid-domain antibody (266: directed against amino acids 13–28 of Aβ). Notably, antibody 266 fails to bind Aβ deposited in CAA vessels or amyloid plaques [31]. Importantly, Ferrer *et al.* [7] noted the presence of CAA and microhemorrhage in the brain of one patient that participated in the Aβ-vaccine trial, even though the parenchymal amyloid appeared lower than expected. Also, Nicoll *et al.* [6] noted that CAA appeared unaffected in the brain of another patient that participated in the Aβ-vaccine trial.

It remains to be determined whether these observations regarding increased CAA and microhemorrhage in transgenic mice are relevant to trials of passive immunotherapy in humans. It should be noted that, in spite of extending the period of immunotherapy to 5 months, there was no discernable loss of the cognitive benefits of immunotherapy in the transgenic mice, all of whom showed increased microhemorrhage. While the observation that antibody 266 does not result in vascular leakage encourages testing of this idiotype, data from the Zurich cohort of the Aβ vaccine trial argue that brain-reactive antibodies may be important for cognitive benefits [32].

Conclusions

Our opinion is that these results suggest that passive immunotherapy against Aβ should proceed with appropriate precautions taken to minimize the risk of hemorrhage (e.g., by excluding patients taking anticoagulants) and instituting measures to detect such hemorrhages if they do occur, irrespective of the antibody specificity or proclivity for microhemorrhage in aged APP-transgenic mice.

List of abbreviations

Aβ : Amyloid-beta.

APP: Amyloid precursor protein

CAA: Cerebral amyloid angiopathy.

IgG1: Immunoglobulin G type 1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DMW treated the mice, performed the behavioral analysis, processed the tissue and performed pathological analyses, and drafted the manuscript. ARojiani evaluated slides and provided expert opinion regarding CAA and microhemorrhage. ARosenthal and SS developed, produced and purified the antibodies used in the studies. MJF performed DNA extraction and PCR for genotyping of the mice. MNG oversees the breeding colony generating mice for the studies, collected samples from the mice and assisted in editing the manuscript. DM conceived the design of the study, guided data interpretation and assisted in editing the manuscript.

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ACCUMULATION OF ANTIBODIES IN THE CENTRAL NERVOUS SYSTEM

By JULES FREUND, M.D.

(From The Henry Phipps Institute, University of Pennsylvania, Philadelphia)

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The purpose of the present paper is to describe the accumulation of antibodies in the central nervous system of rabbits. Information on this subject may throw light upon several important problems: (1) the penetration of protein (globulin) from the blood into the spinal fluid, brain and cord, for antibodies cannot be separated from globulins experimentally, (2) the relationship between the cerebrospinal fluid and the tissue fluid of the brain and spinal cord and (3) the prevention and treatment of certain diseases of the central nervous system by the injection of immune serums.

The present work is a continuation of a series of studies (1) on the antibody content of the serum and organs of rabbits, which have yielded information as to the distribution of agglutinins in the serum, organs and lymph. Agglutinins against typhoid bacilli were selected as antibodies because they can be obtained in high concentration and can be measured easily and with relative accuracy. The rabbit was employed because the titers of the immune serums of rabbits are very much higher than in other small laboratory animals.* The experiments were carried out on actively and passively immunized animals. The active immunization consisted of a series of injections of killed typhoid bacilli into an ear vein. The passively immunized rabbits received one injection of immune serum into the blood stream. The antibody content of the organs was measured by extracting the organs (after grinding with sand) with salt solution and titrating the extracts. For the titration of agglutinins a method was used that is more sensitive than the usual routine method of agglutination. This method consists in centrifugalizing the tubes containing the serum or extract-dilutions and bacteria and reading the results while the tubes are being gently shaken and the sedimented bacteria resus-

* The dog—an animal extensively used in investigations on the central nervous system—does not furnish potent serum. Dogs could have been injected in the passive immunization experiments but by injecting immune serum obtained from rabbits into other rabbits the introduction of foreign protein is avoided.

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pended. This technic was first used by Gathgens (2) in 1906 and has been employed by a number of investigators (3) since that time. For further technical details see previous publications of the present series.

The main results of the work, as already reported, can be summarized as follows: When serum containing antibodies is injected into an ear vein of the normal rabbit, antibodies accumulate in all the organs studied: the liver, spleen, kidney, lung, skin, muscle and uterus (smooth muscle). The rate of accumulation varies in the different organs. The final concentration is reached in the liver, spleen, lung and kidney in less than 10 minutes after the injection of immune serum, but in the uterus and skin only after several hours have elapsed. The antibodies penetrate most slowly into the skin. When the final amount of antibodies is accumulated there is a constant numerical relationship between the antibody content of the blood and organs. On the average, the highest dilution of extract prepared from 1 gm. of liver, spleen, kidney, lung or skin that agglutinated typhoid bacilli was one-tenth of the highest dilution of 1 cc. of serum that agglutinated typhoid bacilli.

Since there are less antibodies in the organs than in similar amounts of blood the question naturally arises whether the antibodies recovered from the organs are due to the blood present in them. That they are not derived mainly from the blood of the organs but from the extravascular part of the tissue is evidenced by the following observations. (1) Lymph obtained by cannulating the lymph ducts of the liver, leg, neck and thoracic duct contains antibodies in higher concentration than the organ extracts. (2) Perfusion does not reduce the antibody content of the skin and uterus. (3) More antibodies can be recovered in the perfusate from the living animal than were present in the blood before perfusion, showing that during perfusion antibodies penetrate from the organs into the blood vessels, an observation recently confirmed by Schwarzmann (4).

The equilibrium between the antibody content of the blood and of the organs can be reached from either the blood or the organs, for an identical relationship will establish itself between the antibody content of the serum and organs when the immune serum is injected either into the blood stream or into the skin.

EXPERIMENTAL

The experiments to be reported here were performed with both actively and passively immunized rabbits for these reasons. Passive immunization offers an opportunity to establish the rate of accumulation of antibodies in the organs and cerebrospinal fluid by examination of the rabbits at different intervals of time after the injection of immune serum. In actively immunized rabbits the titers of the blood and organ extracts are higher and therefore the observations are more striking. The technic of immunization was the same as in the previous work. The immune serum used was fresh, and was obtained and kept under sterile conditions.

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Before describing the experiments, it must be emphasized again that the agglutination tests were made with the aid of centrifugalization, a method that is more sensitive than the routine method of agglutination. Without this technic, the results described in the present study cannot be duplicated.

I. Antibody-Content of the Brain, Spinal Cord and Cerebrospinal Fluid (from Cisterna Magna) of Actively Immunized Rabbits

The technic of these experiments differed from that of the earlier experiments in that urethane was not used for anesthesia because it is said that it promotes the

TABLE I

Agglutinin Titers of the Serum, Spinal Fluid, Brain and Spinal Cord of Actively Immunized Rabbits

Number of rabbit	Serum	Spinal fluid	Brain	Cord
1	1:150,000	1:640	1:2,500	1:1,300
2	1:150,000	1:420	1:600	1:384
3	1:128,000	1:160	—	—
4	1:102,000	1:512	1:576	1:1,200
5	1:102,000	1:256	1:1,500	1:362
6	1:102,000	1:512	—	—
7	1:102,000	1:512	—	—
8	1:102,000	1:512	1:583	1:290
9	1:50,000	1:128	—	—
10	1:32,000	1:80	1:200	1:104
11	1:32,000	—	1:192	1:60
12	1:32,000	1:40	—	—
13	1:26,000	1:48	—	—
14	1:20,000	1:52	—	—
15	1:13,000	1:26	—	—
16	1:3,200	1:5	—	—

passage of substances into the spinal fluid. The animals were narcotized with the minimum amount of ether necessary. Some of the rabbits were bled to death from the left carotid artery; some from the femoral arteries and the descending aorta; the site of bleeding did not influence the results. After the rabbits were bled to death cerebrospinal fluid was removed from the cisterna magna by means of a tuberculin syringe and skin-test needle (gauge 22). After some practice there was no difficulty in obtaining fluid free of blood. The samples of cerebrospinal fluid—in most cases 0.4 cc.—were centrifugalized and the sediment examined under a

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microscope. Samples containing more than one red blood cell in 10 microscopic fields (seen with high dry lens) were rejected. From the brain and spinal cord the meninges were removed very carefully and the ventricles of the brain and central canal of the cord were then opened and carefully rinsed with salt solution. To ascertain whether any cerebrospinal or other fluid containing agglutinins was left on the surface of the organs, the last washing fluid was examined for agglutinins. Some of the washing fluid caused a trace of agglutination, but dilutions 1 in 2, 1

TABLE II

Agglutinin Titers of the Spinal Fluid and of Extracts of the Brain and Spinal Cord of Actively Immunized Rabbits*

Number of rabbit	Spinal fluid	Brain extract	Spinal cord extract
	per cent	*per cent*	*per cent*
1	0.43	1.70	0.87
2	0.28	0.40	0.26
3	0.12	—	—
4	0.50	0.56	1.17
5	0.25	1.47	0.35
6	0.50	—	—
7	0.50	—	—
8	0.50	0.57	0.28
9	0.26	—	—
10	0.25	0.62	0.32
11	—	0.60	0.18
12	0.12	—	—
13	0.18	—	—
14	0.26	—	—
15	0.20	—	—
16	0.20	—	—
Average.....	0.33	0.82	0.49

* The titers are expressed as percentages of the titers of the serum.

in 4 did not agglutinate typhoid bacilli. The washed organs were dried by pressing them lightly between filter papers, weighed, and ground with sea sand alone and with saline. The extracts were centrifugalized at high speed, the sediment discarded, and the supernatant fluid centrifugalized again until it became clear. In recording the results of titration, titer 1:100 means that when 1 gm. of brain was extracted with 9 cc. saline, the extract, diluted ten times, clumped typhoid bacilli and a dilution twice higher—1:200—did not agglutinate typhoid bacilli.

Tables I and II show the following.

1. The cerebrospinal fluid of all the rabbits immunized with killed

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typhoid bacteria contained antibodies. The titer of the cerebrospinal fluid varied with the titer of the serum, the ratio of the titer of the serum to the titer of the cerebrospinal fluid being, on an average, 300:1, or 0.33 per cent. The variation in this numerical relationship exceeded in one case only the limit of accuracy of the method. One should bear in mind, in this connection, that in the agglutination test the serum, cerebrospinal fluid and organ extract are diluted by halves (1 in 100, 1:200).

2. The brain and the spinal cord of all the actively immunized animals contained agglutinins. The titer of the extracts of the brain and cord varied with the titer of the serums, but the numerical relationship between the titers of the serums and organ extracts was not so consistent as the numerical relationship between the titers of the serums and the cerebrospinal fluid. The ratio of the titers of the serums to the titers of the extracts of the brain and of the spinal cord ranged from 100:0.4 to 100:1.7 and from 100:0.18 to 100:1.17 respectively. The average agglutinin titer of brain extract was 0.82 per cent (of the titer of the serum) and that of the spinal-cord extract 0.49 per cent (of the titer of the serum). Therefore extracts prepared from 1 gm. of brain or spinal cord were more potent than the dilution of a similar amount of cerebrospinal fluid.

Summarizing the results of the observations on actively immunized rabbits, the following average numerical relationship was found between the agglutinin titers of the serum, spinal fluid, brain and spinal-cord extracts:

Blood serum	100. per cent
Cerebrospinal fluid	0.33 per cent
Brain extract	0.82 per cent
Spinal-cord extract	0.49 per cent

II. Antibody-Content of the Central Nervous System of Passively Immunized Rabbits

In studies of the distribution of antibodies in the blood and organs of passively immunized animals it is of great advantage to employ immune serums of high titers; therefore only very potent serums were used for passive immunization. The majority of the animals were injected with immune serums of which the titer was 1:64,000. The titer of one of the immune serums was as high as 1:200,000.

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The titer of the serums and the amount injected are given in Table III. The serum was introduced slowly into an ear vein through a skin-test needle (gauge 22), the injection lasting from 3 to 4 minutes. Since it is generally believed that substances in solution injected into a peripheral vein are evenly distributed in the blood stream within 3 minutes, six rabbits were bled to death as early as 15 minutes after the immune serum had been injected. The results of the titrations of the serums, cerebrospinal fluids and organ extracts are tabulated in Tables III and IV.

TABLE III

Agglutinin Titers of the Serum, Spinal Fluid, Extracts of Brain and Spinal Cord of Passively Immunized Rabbits

Number of rabbit	Serum injected		Time between injection of serum and examination	Serum	Spinal fluid	Extract of brain	Extract of spina cord
	Amount	Titer					
1	10 cc.	1:128,000	15 minutes	1:25,000	0	1:80	1:80
2	10 cc.	1:128,000	15 minutes	1:25,000	0	1:50	1:50
3	25 cc.	1:64,000	15 minutes	1:16,000	1:5	1:96	1:29
4	25 cc.	1:64,000	15 minutes	1:24,000	less than 1:2.5	1:240	1:84
5	15 cc.	1:64,000	15 minutes	1:12,000	1:6	1:264	1:180
6	15 cc.	1:64,000	15 minute	1:12,000	less than 1:12	—	—
7	10 cc.	1:128,000	2 hours	1:18,000	1:1.8	1:68	1:18
8	10 cc.	1:128,000	2 hours	1:25,000	—	1:120	1:50
9	10 cc.	1:80,000	2 hours	1:10,000	1:6	1:120	1:80
10	25 cc.	1:64,000	3 hours	1:12,000	1:3.6	—	—
11	20 cc.	1:64,000	3 hours	1:12,000	1:32	—	—
12	15 cc.	1:200,000	4 hours	1:32,000	1:19	1:294	1:126
13	20 cc.	1:64,000	18 hours	1:6,400	1:11	1:29	1:10
14	25 cc.	1:64,000	18 hours	1:3,200	1:8	1:13	1:5
15	20 cc.	1:32,000	20 hours	1:3,200	1:3.2	—	—
16	20 cc.	1:64,000	20 hours	1:4,800	1:18	—	—
17	20 cc.	1:64,000	20 hours	1:3,200	1:10	—	—
18	20 cc.	1:64,000	24 hours	1:3,200	1:11.5	—	—
19	20 cc.	1:200,000	24 hours	1:40,000	1:160	—	—

The tables show that in four rabbits, which were bled beginning 10 to 15 minutes after the injection of immune serum and ending 10 minutes later, the undiluted cerebrospinal fluid failed to agglutinate typhoid bacilli. However, in two other rabbits a very small amount of agglutinins penetrated into the cerebrospinal fluid within that time. The penetration of the antibodies into the cerebrospinal fluid (obtained from the cisterna magna) proceeded at a slow rate, for the high-

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est titers in the cerebrospinal fluid were found only after 15 hours had elapsed following the injection of immune serum. In these animals the titer of the cerebrospinal fluid ranged between 0.10 to 0.40 per cent of the titer of the serum; that is, it was about as high as in actively immunized rabbits.

TABLE IV

Agglutinin Titers of the Cerebrospinal Fluid and of Extracts of the Brain and Spinal Cord of Rabbits Following Intravenous Injection of Immune Serum*

Number of rabbit	Time between injection of serum and examination	Titer of cerebrospinal fluid	Titer of brain extract	Titer of extracts of spinal cord
		per cent	*per cent*	*per cent*
1	15 minutes	0	0.32	0.32
2	15 minutes	0	0.20	0.20
3	15 minutes	0.03	0.60	0.18
4	15 minutes	less than 0.01	1.00	0.35
5	15 minutes	0.05	2.2	1.5
6	15 minutes	less than 0.1	—	—
7	2 hours	0.01	0.38	0.10
8	2 hours	—	0.48	0.20
9	2 hours	0.06	1.20	0.80
10	3 hours	0.03	—	—
11	3 hours	0.27	—	—
12	4 hours	0.06	0.92	0.43
13	18 hours	0.17	0.45	0.15
14	18 hours	0.25	0.40	0.14
15	20 hours	0.10	—	—
16	20 hours	0.37	—	—
17	20 hours	0.31	—	—
18	24 hours	0.36	—	—
19	24 hours	0.40	—	—

* The titers are expressed as percentages of the titers of the serums.

Tables III and IV show that just as in actively immunized rabbits, the extracts of the brain and cord of passively immunized rabbits contained agglutinins. The numerical relationship of the titers of the serums and extracts of brain and cord are very similar to those found in the actively immunized animals, the titer of brain extract being on an average 0.70 per cent and that of the cord 0.71 per cent of the titers of the serum. The variation from the average was more marked than in the actively immunized rabbits.

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It was expected that the accumulation of antibodies in the central nervous system would proceed at a slow rate. This was found true of the penetration of agglutinins into the cerebrospinal fluid. However it had not been expected that the titers of the organ extracts of rabbits examined 15 minutes after the injection of immune serum would be as high as those from rabbits that had been in contact with immune serum for 24 hours. This unexpected observation suggests either that the penetration of antibodies into the brain and spinal cord (tissue fluid of these organs) is as fast as into some organs, such as the spleen, liver and lung and faster than into the uterus and skin, or that the antibodies recovered were due mainly to the blood present

TABLE V

Antibody Titers of Serums and of Extracts of the Perfused Brain of Actively Immunized Rabbits

Number of rabbit	Titer of serum	Titer of brain extract	Titer* of brain
			per cent
1	25,000	270	1.0
2	25,000	180	0.7
3	12,800	770	0.6
4	250,000	1280	0.5
5	128,000	640	0.5

* Expressed as percentages of the titers of the serums.

in the brain and spinal cord. To throw light on this question, I perfused the brain of rabbits of which the blood serum had a high titer in the agglutinin test.

III. Antibody-Content of the Cerebrospinal Fluid, Brain and Spinal Cord after Perfusion

The perfusion experiments were performed as follows. An actively immunized rabbit was lightly narcotized with ether, and about 30 cc. of blood was obtained from the left femoral artery. Then 0.15 gm. of heparin was injected into an ear vein; 3 minutes later the rabbit was bled to death from the femoral arteries and the abdominal part of the descending aorta. Immediately after the bleeding was completed, the brachial arteries and descending part of the aorta were ligated, and Locke solution at 42°C. was introduced into the arch of the aorta with the purpose of perfusing the brain through the vertebral and internal carotid arteries. The

perfusion was usually continued for about 1 hour; about 500 cc. of perfusion fluid was used. The examination of cerebrospinal fluid, and of organ extract was carried out as described above.

Table V shows that perfusion did not diminish the antibody titers of the brain extract or the cerebrospinal fluid. However it was felt that before drawing a conclusion from this observation as to the presence of the antibodies in the extra-vascular brain tissue, it was desirable to obtain evidence of the adequacy of the perfusion experiments. To this end I compared histological sections of the brains of the exsanguinated rabbits with those prepared from rabbits whose brain was perfused. The comparison showed that in the unperfused brains the majority of the blood vessels contained red blood cells whereas in the perfused brains the blood vessels were distended and red blood cells were absent from the majority of them.

Final evidence for the view that the antibodies recovered from the brain by extraction are derived from the extra-vascular part of the tissue would be the demonstration of antibodies in the lymph flowing from the brain. However, no lymph duct draining the brain is known; therefore no direct evidence can be furnished at the present time for this view.

DISCUSSION

The antibody content of the cerebrospinal fluid of normal and diseased human beings and lower animals has been the subject of extensive clinical and experimental investigations. It has been very generally accepted that antibodies do not penetrate from the blood into the cerebrospinal fluid unless the meninges are inflamed. A survey of the literature, however, shows that several authors have reported the presence of antibodies in the cerebrospinal fluid without inflammation.

Hektoen and Carlson (5) found opsonins but no haemagglutinins in the cerebrospinal fluid of actively or passively immunized dogs. Becht and Greer (6) could not demonstrate agglutinins in the cerebrospinal fluid of rabbits immunized with typhoid vaccine. Kafka (7) found traces of hemolysins and bacterial agglutinins in the cerebrospinal fluid of immunized dogs. Starkenstein and Zitterbart (8) reported that only undiluted cerebrospinal fluid of dogs agglutinated typhoid bacilli, although the titer of their serum was as high as 1:10,000.

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There are two reports in the literature on the relative titers of tetanus antitoxin in the serum and cerebrospinal fluid. Ransom (9), working in von Behring's laboratory, compared the antitoxin titers of the serum and of the cerebrospinal fluid of one very highly immunized horse. He found that the ratio of the antitoxin titer of the serum of this animal to that of the cerebrospinal fluid was 100:0.4. Lemaire and Debre (10), who studied the effect of morphine upon the permeability of the meninges, reported that in dogs injected with tetanus antitoxin the ratio of the titer of the serum to that of the cerebrospinal fluid was 100:0.2.

The experiments performed in the present study show clearly that antibodies are present in the cerebrospinal fluid of actively or passively immunized animals without inflammation of the meninges. The possible objection that the antibodies demonstrated were due to inflammation or contamination of the cerebrospinal fluid with blood as a result of faulty technic can be met as follows. (1) The specimens of cerebrospinal fluid did not contain red blood cells at all or only in a negligible number. (2) There was a constant numerical relationship of the titers of serums to the titers of cerebrospinal fluids. (3) In passively immunized rabbits the antibody titer of the cerebrospinal fluid increased during the first 15 hours following the injection of immune serum, although the titer of the blood decreased during this time.

It is pertinent to inquire whether the central nervous system of the rabbits used in these experiments was free of pathological changes. McCartney (11) at the suggestion of Flexner, examined the brains of a large number of apparently healthy rabbits and found histological evidence of meningo-encephalitic lesions in more than 50 per cent. His observation is in conformity with those made in other laboratories (Bull (12), Oliver (13)).

The brains of rabbits employed in the present work were not examined histologically, but it is reasonable to assume that the lesions found by McCartney occur in the brains of our stock rabbits.

McCartney, Bull and Oliver did not study the spinal fluid of their rabbits and therefore it is not known whether the lesions found by them in the brain are associated with a large number of leucocytes or with other signs of inflammation in the cerebrospinal fluid. Although the spinal fluid of the rabbits employed in the present work was free from an abnormal number of leucocytes, the possibility cannot be excluded that meningo-encephalitic lesions so prevalent in apparently normal

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rabbits did not influence the accumulation of antibodies in the central nervous system in some of them. However antibodies were found in the spinal fluid, brain and spinal cord in all of the actively and all of the passively immunized rabbits 2 hours after passive immunization. It hardly seems possible that the accumulation of antibodies in the central nervous system of all the rabbits could have been due solely to the presence of meningo-encephalitis.

The reasons for the general belief that antibodies do not penetrate from the blood into the cerebrospinal fluid without the inflammation of the meninges are probably the following. (1) The antibody titer of the cerebrospinal fluid is relatively low, as would naturally follow from the circumstance that the antibody titers of the serums of animals examined in the course of the reported studies were not high. (2) A large number of studies dealt with normal hemolysins, whose titer in the serum is very low.

Amoss and Eberson (14) reported that "agglutinins were not found in the spinal fluid of normal monkeys which had received antimeningococcic serum intravenously." This observation can be readily explained by the experiments on rabbits reported here, which show a ratio of 300:1 between the titers of the serum and of the spinal fluid; whereas the serum of the monkey in the experiment of Amoss and Eberson contained only 100 units of agglutinins per cubic centimeter, and the amount of agglutinins present in the spinal fluid was, therefore, too small to be detected. Flexner, Clark and Amoss (15) found that "it is unusual for the neutralizing principles to be contained in the cerebrospinal fluid during convalescence from epidemic poliomyelitis," although neutralizing principles are present in the blood. It is, however, not probable that the serums of the convalescents contained the neutralizing principles or antibodies in quantities that could be demonstrated in three-hundred-fold dilution.

Although, as my experiments show, antibodies penetrate from the blood into the spinal fluid of rabbits even without inflammation, the fact remains that the antibody content of the spinal fluid is very small. The results of numerous clinical and experimental observations have shown the effectiveness of sterile inflammation and of injecting immune serum into the spinal fluid in raising the antibody content of the spinal fluid (Flexner). In experiments to be reported later this finding has been reobtained in rabbits.

Since antibodies are found in the globulin fraction of the serum, behave in many respects like globulins and may be expected to follow the distribution of globulins, it is interesting to compare the ratio of the antibody titers of the serum and the cerebrospinal fluid on

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the one hand with the ratio of the globulin of the serum and that of the cerebrospinal fluid on the other hand. Mestezrat (16) stated that the globulin content of the cerebrospinal fluid is about 0.019 gm. per 100 cc., and it is said that 100 cc. of serum contains on an average 2.5 gm. of globulin. These data were obtained from the serum and cerebrospinal fluid of man, but it is possible that the globulin content of the serum and cerebrospinal fluid in the rabbit is at least of similar magnitude if not almost equal to that found in human beings. The ratio of globulin content of the serum to that of the cerebrospinal fluid,

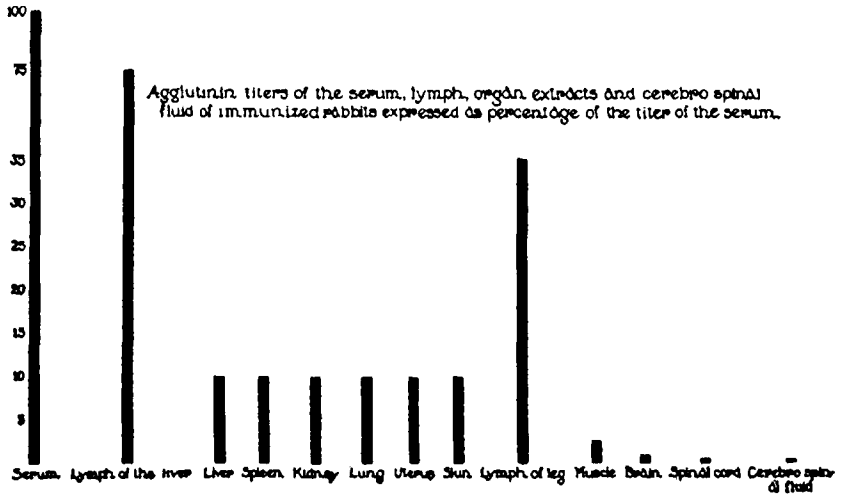

CHART 1

100:0.76, nearly equals that of the respective antibody titers, 100: 0.30. Considering the accuracy of the technic of agglutination tests a closer agreement can hardly be expected. Therefore it can be said there is some parallelism between the antibody titers of the serum and cerebrospinal fluid on the one hand and the globulin content of these fluids on the other hand.

These observations have an obvious bearing on the serum therapy of the central nervous system. They show that antibodies do accumulate in the tissue of the brain, spinal cord and cerebrospinal fluid even if the immune serum is not injected into the central nervous sys-

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tem; furthermore, that the antibody content of the central nervous system can be estimated by titration of the blood.

It is important to ascertain whether antibodies accumulate in the tissues of the central nervous system at a faster rate when the immune serum is injected into the spinal fluid instead of the peripheral blood stream. This question is being investigated and will be the subject of a subsequent publication.*

In the previous studies it was found that the agglutinin titers of the extracts of the spleen, liver, lung, kidney, uterus and skin are about the same and on the average ten times lower than that of the serum (extract of 1 gm. of organ compared with dilution of 1 cc. of serum). The titer of extracts of the muscles of the leg is lower than those of the other organs examined, varying from 1 to 5 per cent expressed as percentage of the titer of the serum. In contrast to these organs the brain and cord yield extracts that contain antibodies in very low titer, less than 1 per cent of the titer of the serum. (See Chart 1.)

CONCLUSIONS

1. Antibodies can be extracted from the brain and spinal cord of rabbits actively or passively immunized with typhoid bacilli.

2. The titers of the antibodies in the extracts of brain and cord depend upon the titer of the blood serum. In actively immunized rabbits the following numerical relationships exist between the titers of the serum and of these organ extracts: The ratio of the titer of the serum is to the titers of extract of brain and of the spinal cord about as 100 is to 0.8; the titer of the serum is to the titer of the cerebrospinal fluid as 100 is to 0.3. In passively immunized rabbits the titer of the serum is to the titer of brain and spinal-cord extract as 100 is to 0.7.

3. The antibodies recovered from the brain are not due to the presence of blood in it for perfusion of the brain does not reduce its antibody content appreciably.

4. Antibodies penetrate into the spinal fluid from the blood even in the absence of inflammation of the meninges. When the penetration is completed the following numerical relationship exists between the titer of the serum and that of the cerebrospinal fluid: 100 to 0.25.

* More general discussion of the literature will be published in a subsequent paper.

5. The penetration into the cerebrospinal fluid of antibodies injected intravenously proceeds at a slow rate, being completed only several hours after the immune serum has been injected. The penetration of antibodies into the tissue of the brain occurs at a very rapid rate. It is completed within 15 minutes.

6. It is very unlikely that when the immune serum is injected intravenously the antibodies reach the brain tissue by way of the cerebrospinal fluid, for (1) the antibody titer of the cerebrospinal fluid is lower than that of the brain extract, and (2) antibodies penetrate faster into the tissue of the brain than into the cerebrospinal fluid.

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Aβ N-terminal-end specific antibody reduced β-amyloid in Alzheimer-model mice

Yuko Horikoshi[a,b], Takashi Mori[c], Masahiro Maeda[b], Nóriaki Kinoshita[b], Kumiko Sato[a], Haruyasu Yamaguchi[a,*]

[a] *Gunma University School of Health Sciences, Maebashi, Gunma 371-8514, Japan*
[b] *Immuno-Biological Laboratories Co., Ltd. (IBL), Fujioka, Gunma 375-0005, Japan*
[c] *Institute of Medical Science, Saitama Medical Center/School, Kawagoe, Saitama 350-8550, Japan*

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease with memory dysfunction that is causing serious medical problems in modern society. For the fundamental treatment of AD, an amyloid β protein (Aβ) vaccine is considered to be the most potent candidate. To cure AD, we developed Aβ N-terminal-end specific monoclonal antibody named 82E1, which does not cross-react with full-length Aβ precursor. Passive intraperitoneal administration of 82E1 markedly reduced total plaque area (Aβ burden) in the Tg2576 mouse brains. This was confirmed by the ELISA measurement of insoluble Aβ in the brain homogenates. The density of diffuse plaques, which increases in the late stage, was markedly reduced by the administration of 82E1, suggesting that the reduction of the Aβ burden was due to the prevention of newly developed diffuse plaques. Above results provide an insight into the further therapeutic intervention in AD with few adverse effects.

Keywords: Aβ; Administration; Alzheimer; Antibody; APP; ELISA; Immunohistochemistry; Tg2576

Alzheimer's disease (AD) is one of the most serious neurodegenerative diseases. Deposition of amyloid β protein (Aβ), a major component of senile plaque amyloid, promotes neurofibrillary changes and synaptic loss, resulting in dementia in AD. In 1999, Schenk et al. [1] have reported that vaccination with Aβ1-42 effectively reduced cerebral β amyloid deposition in Aβ precursor (APP) transgenic mouse brains. The clinical trial with Aβ1-42 (AN-1792) was discontinued due to the adverse effect, namely neuroinflammation [2]. In a subsequent paper, intraperitoneal injection of Aβ antibody has also reduced β amyloid deposition in the transgenic mice, showing the significant effect of passive immunization [3,4]. Since the antibodies used in the above studies reacted with full-length APP as well as Aβ, the administration of these antibodies would have potential adverse effects in clinical applications. Thus, it is accumulating interest to reduce adverse effects by further modification of Aβ antibodies.

Recently, we developed a monoclonal antibody, named 82E1. It has high specificity and high reactivity against Aβ N-terminal-end, and therefore does not cross-react with full-length APP [5]. Here, we examined the effect of passive immunization with 82E1 on Tg2576 mouse brains.

Materials and methods

All animal protocols were in accordance with the guidelines of the Animal Use Ethics Committee of the Saitama Medical Center/School

* Corresponding author. Fax: +81 27 220 8946.
E-mail address: yamaguti@health.gunma-u.ac.jp (H. Yamaguchi).

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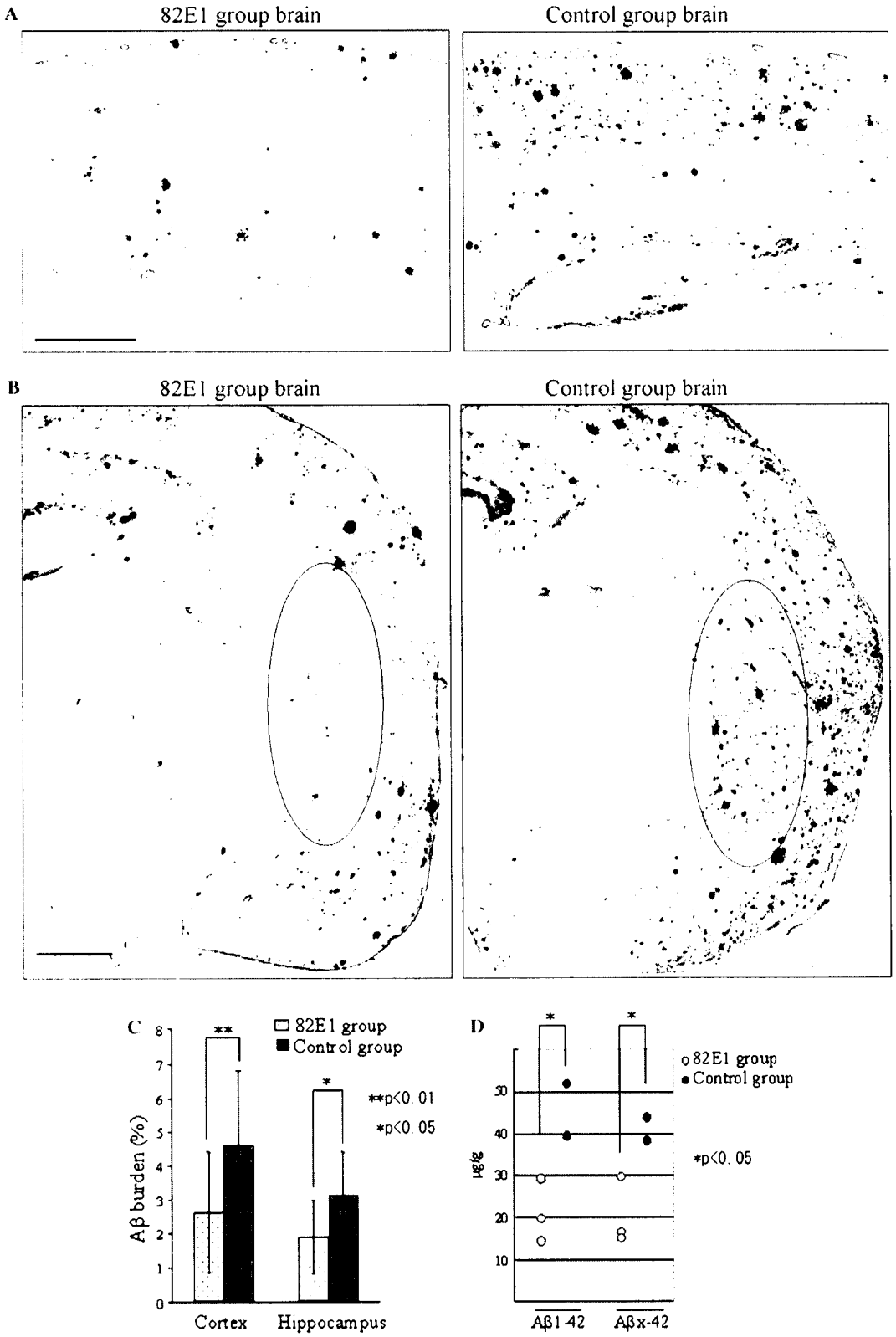

Fig. 1. (A) Aβ burden was reduced by administration of 82E1 antibody when compared to that in the control group; Aβ42 staining. Bar = 500 μm. (B) Senile plaques in basal ganglia (in the circle) were absent in the 82E1 group; Aβ42 staining. Bar = 500 μm. (C) Aβ burden (Aβ42 positive plaque area) of the cortex ($p < 0.01$) and hippocampus ($p < 0.05$)was significantly lower in the 82E1 group than in the control group. (D) Insoluble Aβ1-42 and Aβx-42 were significantly reduced in the 82E1 group based on ELISA measurements. $^{*}p < 0.05$, $^{**}p < 0.01$.

and NIH guidelines [DHHS publication No. (NIH) 85-23, revised 1985].

Purified Aβ N-terminal-end specific monoclonal antibody 82E1 (1 mg/ml in PBS) was administered once a week by intraperitoneal injection at a dose of 10 mg/kg to 16-month-old Tg2576 mice (82E1 group, $n = 3$,), while PBS was administered for the control group of Tg2576 mice (control group, $n = 2$) [3]. After 12 times administrations for over 3 months, blood sample was collected transcardially for the ELISA measurement of soluble Aβ in the plasma level under the deep anesthesia by sodium pentobarbital (25 mg/kg), and mice were euthanized by transcardial perfusion of 100 ml of 10 U/ml heparin in saline. The left cerebral hemisphere was fixed in 4% buffered formaldehyde solution and embedded in paraffin. Serial sections (5-μm in thickness) of the left cerebral hemisphere at 10 predetermined coronal planes separated by 1-mm intervals were sequentially cut and immunostained with Aβ40 and Aβ42 end specific polyclonal antibodies (2 μg/ml; IBL, Japan) after brief formic acid pretreatment using an ABC *Elite* kit (Vector, USA). Additionally, to examine the distribution of 82E1 into the brain parenchyma, one mouse in the 82E1 group was intraperitoneally administered by the biotinylated 82E1 1 day before sacrifice. The distribution of biotinylated 82E1 was visualized using an avidin-horseradish peroxidase in 4% buffered formaldehyde-fixed vibratome sections. Microglia in paraffin sections were visualized using Iba-1 antibody (2 μg/ml; Wako, Japan).

Images of 10 selected sections at fixed intervals from the cortex and five from hippocampus of each mouse were acquired using an Olympus BX60 microscope with an attached digital camera system (DP-50, Olympus, Japan), and the digital image was routed into a Windows PC for quantitative analysis using SimplePCI software (Compix, Imaging Systems, USA). Aβ40 and Aβ42 burdens were presented as the percentage of immunolabeled area captured (positive pixels) divided by the full area captured (total pixels).

TBS-insoluble Aβ was extracted using 6 M-guanidine-HCl [6] from the right frontal quarter of brains and then used for the ELISA measurement together with plasma. Aβ1-40 and Aβ1-42 were measured by combinations of 82E1 with Aβ40 or Aβ42 C-terminal end-specific antibodies 1A10 and 1C3, respectively, as described previously [5]. Aβx-40 and Aβx-42 were also measured by combinations of 12B2, Aβ middle portion antibody, with 1A10 or 1C3 [5].

Results and discussion

We observed the effect of 82E1 administration. Immunohistochemistry for Aβ42 showed a smaller number of plaques in the 82E1 group than in the control group (Figs. 1A and B). In the basal ganglia, where Aβ deposit occurred later than that in the cortex and hippocampus, senile plaques were markedly less in the 82E1 group than in the control group (Fig. 1B). This reduction was confirmed by image analysis (Fig. 1C). The Aβ burden was significantly reduced in the 82E1 group compared with that in the control group for both Aβ40 (30% reduction, $p < 0.05$, in cortex; 40% reduction, $p < 0.05$, in hippocampus) and Aβ42 (43% reduction, $p < 0.01$ in cortex; 40% reduction, $p < 0.05$, in hippocampus) (Fig. 1C).

Aβ ELISA analysis of TBS-insoluble fraction was consistent with the findings on immunohistochemical studies. Significant reduction of Aβ42 was found in the 82E1 group. The 82E1 group showed about 50% less Aβ1-42 and Aβx-42 than the control group (Fig. 1D). Aβ40 tended to reduce in the 82E1 group compared to that in the control group, although its reduction did not reach a significant level (data not shown). The Aβ level in plasma was not significantly different between the 82E1 and control groups (data not shown).

Although we attempted to visualize the distribution of biotinylayed 82E1 antibody into the brain parenchyma, the antibody employed in this study failed to across into the brain parenchyma (data not shown).

From 15 to 23 months in the Tg2576 brains, Aβ deposits increased markedly in number and burden, as shown in the control group [7]. Numbers of diffuse plaques rapidly increase in this period. However, the 82E1 group brains showed fewer diffuse plaques than we expected. Thus, we concluded that the 82E1 administration reduced senile plaques, especially diffuse plaques, in Tg2576 mice.

Bard et al. [4] suggested that clearance of the Aβ deposition was induced by phagocytosis of activated microglia, and that activation of microglia was mediated by Fc receptor. In our study, activated microglia were similarly found in both the 82E1 and control groups (data not shown).

These findings suggest that antibody administration reduced the Aβ burden without microglial activation. In fact, the Aβ clearance mechanism in the brain was independent of microglial activation [8,9]. We could not confirm the distribution of 82E1 antibody into the brain parenchyma. Therefore, our findings agree with the report that the Aβ antibody exists in the peripheral blood and reduces the Aβ burden by altering the central nervous system and plasma Aβ clearance [10].

The current study demonstrated that administration of antibody against the N-terminal of Aβ, like 82E1, effectively reduces plaques. The 82E1 does not react with full-length APP. In the brain, APP exists in neuronal cells, thus an antibody that reacts with full-length APP might bind to normal cells and promote inflammation. Thus, 82E1 might be a good therapeutic agent.

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Brief Communication

Reversible Memory Loss in a Mouse Transgenic Model of Alzheimer's Disease

Linda A. Kotilinek,[1] Brian Bacskai,[2] Marcus Westerman,[1] Takeshi Kawarabayashi,[3] Linda Younkin,[3] Bradley T. Hyman,[2] Steven Younkin,[3] and Karen H. Ashe[1]

[1]Departments of Neurology and Neuroscience, University of Minnesota, Minneapolis 55455, [2]Department of Neurology, Massachusetts General Hospital East, Charlestown, Massachusetts 02129, and [3]Department of Neuroscience, Mayo Clinic, Jacksonville, Florida 32224

Alzheimer's disease (AD) is a neurodegenerative condition, believed to be irreversible, characterized by inexorable deterioration of memory and intellect, with neuronal loss accompanying amyloid plaques and neurofibrillary tangles. In an amyloid precursor protein transgenic mouse model, Tg2576, little or no neuronal loss accompanies age-related memory impairment or the accumulation of Aβ, a 40–42 aa polypeptide found in plaques. Recently, we have shown inverse correlations between brain Aβ and memory in Tg2576 mice stratified by age (Westerman et al., 2002). Broadening the age range examined obscured this relationship, leading us to propose that small, soluble assemblies of Aβ disrupt cognitive function in these mice. Here we show that memory loss can be fully reversed in Tg2576 mice using intraperitoneally administered BAM-10, a monoclonal antibody recognizing the N terminus of Aβ. The beneficial effect of BAM-10 was not associated with a significant Aβ reduction, but instead eliminated the inverse relationship between brain Aβ and memory. We postulate that BAM-10 acts by neutralizing Aβ assemblies in the brain that impair cognitive function. Our results indicate that a substantial portion of memory loss in Tg2576 mice is not permanent. If these Aβ assemblies contribute significantly to memory loss in AD, then successfully targeting them might improve memory in some AD patients.

Key words: Alzheimer's disease; transgenic; behavior; Aβ; monoclonal antibodies; memory

The Tg2576 transgenic mouse model of Alzheimer's disease (AD), which overexpresses a mutant form of amyloid precursor protein (APP), $APP_{K670/671L}$, linked to early onset familial AD, develops amyloid plaques and progressive cognitive deficits (Hsiao et al., 1996). In these mice, Aβ begins to rise rapidly at ~6 months, coincident with the appearance of detergent-insoluble Aβ (Kawarabayashi et al., 2001), and memory ability declines progressively thereafter (Westerman et al., 2002). Punctate, cored plaques are present in 7- to 8-month-old mice; mature, diffuse plaques appear at ~12 months of age (Kawarabayashi et al., 2001). Descriptive characterizations of the relationship between memory and Aβ in Tg2576 mice (Westerman et al., 2002), along with active Aβ immunization studies in Tg2576 and other APP transgenic mice (Janus et al., 2000; Morgan et al., 2000), have demonstrated that Aβ is necessary and sufficient to disrupt memory and have implicated a soluble Aβ assembly rather than the accumulation of Aβ or amyloid plaques per se.

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Correspondence should be addressed to Karen H. Ashe, Department of Neurology, Mayo Mail Code 295, 420 Delaware Street Southeast, Minneapolis, MN 55455. E-mail: hsiao005@umn.edu.

T. Kawarabayashi's present address: Department of Neurology, Okayama University Graduate School of Medicine, Okayama, 700-8558 Japan.

There have been no studies addressing whether the deleterious effects of Aβ on cognitive function are permanent. Tg2576 mice at 16 months of age with mature plaque deposition show no neuronal or synaptic loss (Irizarry et al., 1997), leading us to surmise that cognitive impairment in these mice might be attributable to neuronal dysfunction rather than neuronal degeneration. Based on previous studies of the relationship between Aβ and memory in Tg2576 mice (Westerman et al., 2002), we hypothesized that if cognitive deficits related to toxic Aβ assemblies occur primarily in the absence of structural damage, then passive administration of antibodies to Aβ might rapidly reverse learning and memory deficits by neutralizing one or more critical Aβ species, thereby restoring normal cognitive function. To focus our evaluation on alterations in cognitive function that occur before plaque deposition, we tested Tg2576 mice at 9–11 months after the appearance of detergent-insoluble Aβ, but preceding the accumulation of abundant mature amyloid plaques (Kawarabayashi et al., 2001). At this age, punctate deposits are present but are rare and difficult to quantify meaningfully. Because passive immunization affects molecular targets more rapidly and selectively than active immunization, we chose passive immunization as a tool to clarify the molecular mechanism by which memory loss occurs in Tg2576 mice.

MATERIALS AND METHODS

Mice and behavioral testing. Forty-three female Tg2576 mice, positive for the HuAPP695.K670N/M671L transgene in a hybrid C57BL/6/SJL background (Hsiao et al., 1996), were longitudinally tested twice at 9–11 months of age; a total of 17 Tg2576-positive mice (10 female, 7 male) were longitudinally tested at 2 and 8 months of age, and 10 littermates negative for the transgene (7 female, 3 male) were tested at 3 months of

age, in the reference memory version of the Morris water maze (Morris, 1984), as described previously (Westerman et al., 2002).

In the longitudinal experiment involving 9- to 11-month-old mice, a baseline assessment of the cohort was obtained immediately before immunization, first in the visible-platform version of the water maze (3 d, eight trials per day) followed by hidden-platform testing (9 d, four trials per day). The spatial memory for the platform position was evaluated in 1 min probe trials administered at the beginning of days 4, 7, and 10 of hidden platform testing. Mice were allocated to the two treatment groups that were counterbalanced on the basis of the mean of the three baseline probe scores. All cues were changed, and the platform position was shifted to the opposite quadrant during subsequent retesting of immunized mice performed 11–12 d after the termination of the baseline water maze test. Only a hidden-platform version of the water maze test was performed. The order of testing mice from different experimental groups was random, and the experimenters were unaware of the treatment group. Eight mice that were unable to learn the visible-platform test or be led out of the pool with an escape scoop were removed from the experiment, a proportion consistent with previous studies (Westerman et al., 2002). One mouse died during baseline testing, before immunization, and another mouse died 1–2 hr after the final BAM-10 injection, reducing the final control (IgG) and treatment (BAM-10) group sizes to 17 and 16, respectively. The latter mouse showed no signs of illness at the time of injection, making it likely that the acute death was related to a traumatic injection rather than to encephalitis.

Seventeen naive Tg2576 mice, along with 10 transgene-negative littermates, were also tested at 2 and 3 months of age, respectively, using the same protocol, except that these mice were prehandled before testing. Prehandling consisted of performing preparative maneuvers resembling procedures used during testing 8–10 times during the 2–3 weeks before actual testing. Previous cross-sectional studies of spatial reference memory during the lifetime of Tg2576 mice in the C57BL/6/SJL background have shown no differences between Tg2576 mice at <6 months of age and nontransgenic littermates at ≤20 months of age (Westerman et al., 2002). For this reason, we chose to compare Tg2576 mice at 9–11 months of age with younger Tg2576 mice and nontransgenic littermates. At 8 months of age, the 17 Tg2576 mice were allocated into two treatment groups counterbalanced on the basis of mean probe scores at 2 months of age and gender, treated with BAM-10 or nonspecific IgG, retested in the water maze beginning at 8.3 months of age, and killed at 8.7 months of age.

Antibody selection and administration. BAM-10 (Sigma, St. Louis, MO) is a mouse monoclonal antibody recognizing Aβ(1–12). BAM-10 was chosen on the basis of its ability to bind Aβ *in vivo*. Because not all antibodies bind to Aβ in its native configuration (B. Bacskai and B. T. Hyman, personal communication), we used multiphoton microscopy, an *in vivo* imaging method with ~1 μm resolution, to evaluate the effectiveness of BAM-10 antibody in living Tg2576 mice. We purified, concentrated, and labeled BAM-10 with fluorescein, and applied 5–10 μl of a 1 mg/ml solution directly to the cortical surface of 25-month-old Tg2576 mice. We then visualized the fluorescence as described previously (Bacskai et al., 2001), readily imaging both senile plaques in the neuropil and amyloid angiopathy in the living mouse brain. The *in vivo* immunofluorescent signal colocalized with thioflavine S staining in cored plaques and in amyloid angiopathy, as well as revealing nonthioflavine S diffuse deposits (data not shown). Diffuse but not cored deposits were reduced by 53% after 3 d in BAM-10-treated mice, an effect similar to that obtained using another antibody recognizing the N terminus of Aβ, 10D6 (Bacskai et al., 2001) (data not shown).

Antibodies were administered intraperitoneally beginning 4–5 d after the last day of baseline water maze pretesting in the longitudinal experiment involving 9- to 11-month-old mice, and 7 d before water maze testing in the experiment involving 8-month-old mice. One group of Tg2576 mice received BAM-10 ascites lacking sodium azide preservative; the other group if mice received IgG (Sigma). Animals received 0.5 mg of antibody on days 1, 6, and 12 and received 0.25 mg of antibody on day 4. Mice were killed on the last day of behavioral testing, 5 d after the last dose.

Serum antibody titers. BAM-10 serum titers were measured using an adaptation of methods described previously (Schenk et al., 1999). Microtiter ELISA plates (Costar, Cambridge, MA) were coated with 1 μg of aggregated Aβ42 (American Peptide Company, Sunnyvale, CA) in PBS, pH 8.5, and blocked with 1% BSA (Sigma) in PBS, pH 7.4. Plates were washed with wash buffer (PBS, 0.05% Tween 20), and threefold serial dilutions (1:50 to 1:1350) of mouse serum in PBS, 1% BSA, 0.05% Tween 20, and 0.02% sodium azide were incubated overnight at 4°C. Plates were washed and incubated for 1 hr at room temperature in a 1:10,000 dilution of sheep anti-mouse HRP conjugate (Jackson ImmunoResearch, West Grove, PA) in PBS, 0.05% Tween, and 0.1% BSA. Plates were washed and developed with 3,3′5,5′ tetramethylbenzidine (1-Step Slow TMB; Pierce, Rockford, IL). The reaction was stopped with an equal volume of 1 M H_2SO_4, and plates were read at 450 nm. Optical densities (ODs) of equivalently diluted normal mouse serum were subtracted from test sera to obtain the net OD. The antibody titer was defined as the dilution of serum yielding a net OD that was 50% of the maximal signal for that specimen.

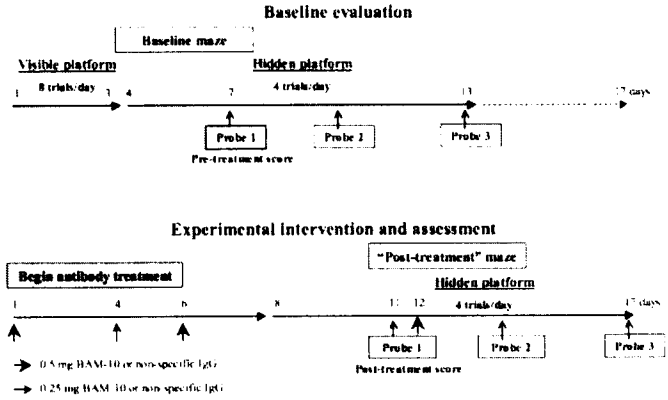

Figure 1. Longitudinal experimental design using Tg2576 mice to determine whether memory loss, once present, can be restored. Spatial reference memory was measured, using the Morris water maze (Morris, 1984), immediately before and after intraperitoneal administration of BAM-10, a monoclonal antibody recognizing the N terminus of Aβ.

Aβ measurements. Aβ was measured by ELISA using the 3160 capture antibody described previously (Kawarabayashi et al., 2001).

RESULTS

BAM-10 restores spatial learning and memory

We measured spatial reference memory, using the Morris water maze (Morris, 1984), immediately before and after treatment with BAM-10 (Fig. 1). Training trials were delivered in blocks of four trials per day, and probe trials were performed on the mornings after the 12th, 24th, and 36th training trials. When memory impairment in Tg2576 mice first emerges at 9–11 months of age, it can be overcome with extensive training, making Tg2576 mice appear to be comparable with nontransgenic littermates at the end of training (Westerman et al., 2002). The slower rate of learning of Tg2576 mice at this particular age is easily detectable at the beginning of training, in the earlier probe scores (Westerman et al., 2002). The first probe scores were therefore used to assess treatment effects in this study.

Animals were assigned to BAM-10 or IgG groups after the baseline maze, counterbalancing for probe scores. The percentage of time spent by mice in the target quadrant during the baseline test in the BAM-10 and IgG groups was not significantly different. Mice received three injections of either 0.5 mg of BAM-10 or nonspecific mouse IgG (days 1, 6, and 12 of the experiment) with a booster of 0.25 mg on day 4. BAM-10 serum titers, measured using an adaptation of methods described previously (Schenk et al., 1999), ranged from 1:100 to 1:1100 at 5 d after the last dose. Performance in the water maze was reassessed beginning on day 8. The two groups showed significant differences in changes in performance between baseline and post-treatment tests [$p = 0.03$ by t test or by two-way (treatment-by-test session) ANOVA with repeated measures], indicating a significant effect of treatment with BAM-10 (Fig. 2*a*).

Because not all mice deteriorate at the same rate, a minority of

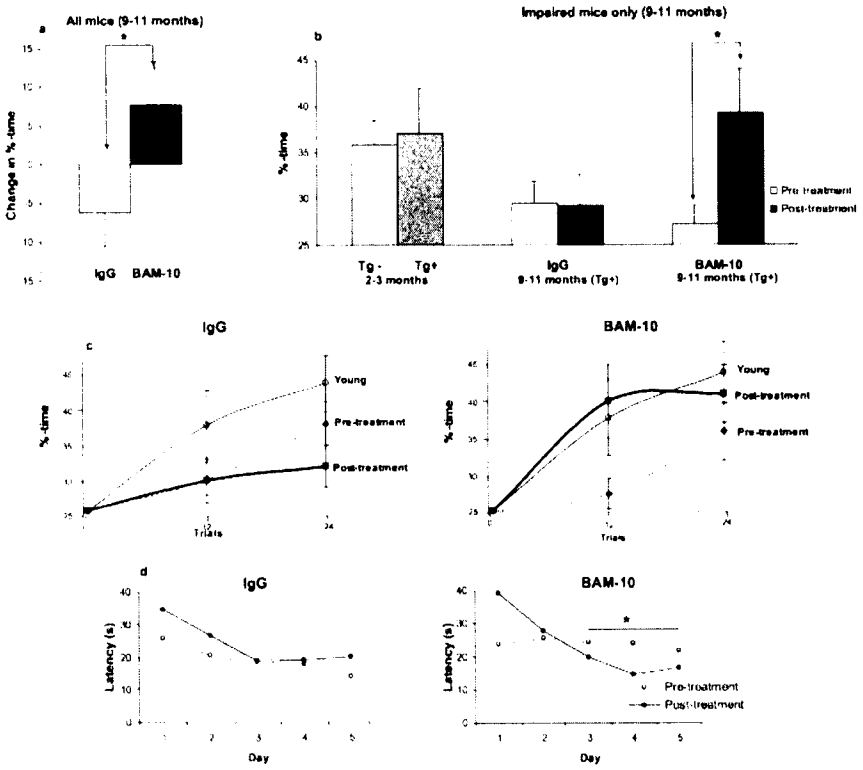

Figure 2. Spatial reference learning and memory in 9- to 11-month-old Tg2576 mice before and after treatment with BAM-10 antibody. The change in retention of spatial memory occurring as a result of receiving BAM-10 or IgG antibodies intraperitoneally was measured by subtracting baseline scores from post-treatment scores to obtain the change in percentage of time spent in the target quadrant (*Change in % time*). *a*, Mice 9–11 months of age receiving BAM-10 antibody showed significantly greater improvement than mice receiving nonspecific IgG (*$p = 0.03$ by t test; IgG, $n = 17$; BAM-10, $n = 16$). *b*, In mice that were impaired at baseline (<40% of the time spent in the target quadrant), those receiving BAM-10 antibody also showed significantly greater improvement than those receiving nonspecific IgG (*$p = 0.04$ by two-way ANOVA with repeated measures; IgG, $n = 13$; BAM-10, $n = 14$). Post-treatment performance of impaired mice receiving BAM-10 antibody was significantly higher than baseline performance (*$p = 0.01$ by paired t test) and was similar to that of 2-month-old Tg2576 mice ($n = 17$) and 3-month-old nontransgenic littermates ($n = 10$). *c*, BAM-10, but not nonspecific IgG, restored the retention learning curve of 9- to 11-month-old Tg2576 mice to resemble that of 2-month-old (*Young*) Tg2576 mice. *d*, Acquisition of spatial reference memory improved in impaired mice receiving BAM-10 antibody, with significantly reduced mean escape latencies on days 3–5 (*$p = 0.04$ by paired t test), but not in mice receiving nonspecific IgG. There was a significant treatment-by-training session (baseline vs post-treatment) interaction ($p = 0.03$ by two-way ANOVA with repeated measures).

9- to 11-month-old mice showed superior performance (>40% of time in the target quadrant) comparable with that of the top third of nontransgenic mice. We subsequently segregated the mice on the basis of baseline scores into impaired (<40% of time in the target quadrant) and superior (>40% of time in the target quadrant) groups. To address the question of whether BAM-10 reversed deficits, we compared the magnitude of the change between baseline and post-treatment scores in impaired mice only. There was a significant treatment-by-test session (baseline vs post-treatment) interaction in impaired mice ($p = 0.04$ by two-way ANOVA with repeated measures) (Fig. 2*b*). Post-treatment scores in impaired mice receiving IgG showed essentially no change relative to baseline scores. In contrast, impaired mice receiving BAM-10 demonstrated significantly improved scores ($p = 0.01$ by paired t test) (Fig. 2*b*). Remarkably, post-treatment memory ability in BAM-10-treated mice was similar to that of nontransgenic mice tested at 3 months or transgenic mice tested at 2 months, before the onset of memory loss (Fig. 2*b*), indicating that the memory deficits in 9- to 11-month-old Tg2576 mice were reversed and memory was fully restored with BAM-10. These results are supported by comparing the learning curves of retention for BAM-10 and nonspecific IgG treatments in Tg2576 mice (Fig. 2*c*). BAM-10 restored the learning curve in 9- to 11-month-old mice to resemble that of 2-month-old Tg2576 mice.

The restorative effects of BAM-10 were also evident when acquisition of spatial reference information was examined in impaired mice. We compared mean escape latencies on days 3–5, because differences in the performance of Tg2576 mice at this age were most pronounced during this phase of training, consistent with the maximal sensitivity of the first probe trial on day 4. There was a significant treatment-by-training session (baseline vs post-treatment) interaction for mean escape latencies ($p = 0.03$ by two-way ANOVA with repeated measures). Mean escape latencies measured before and after IgG administration showed no significant differences [mean difference, 2.5 sec; 95% confidence interval (CI), −2.8 to 7.8 sec; $p = 0.32$ by paired t test] (Fig. 2*d*). In contrast, mean escape latencies after BAM-10 treatment improved significantly (mean difference, −6.3 sec; 95% CI, −0.3 to −12.2 sec; $p = 0.04$ by paired t test) (Fig. 2*d*). We also observed longer escape latencies on day 1 of the post-treatment test in both groups of mice, suggesting a retest effect in which mice exhibited retention of spatial information from the baseline water maze test. The retest effect rapidly extinguished with retraining in the BAM-10-treated mice but not in the IgG-treated mice. The beneficial effects of BAM-10 were apparent within 11 d of the first antibody dose, the smallest time interval we could measure in this study, given the 8 d elapsing between the first dose and the commencement of retesting in the water maze and the 3 d training interval until the first probe trial.

No significant changes were observed in Aβ levels

After behavioral testing, the brains of 19 IgG-treated mice and 18 BAM-10-treated mice were sequentially extracted first in Tris-buffered saline (TBS), then in 2% SDS, and finally in 70% formic acid (Kawarabayashi et al., 2001). Aβ40 and Aβ42 were then analyzed in each fraction by sandwich ELISA. This analysis showed that the improved performance in mice treated with BAM-10 was not associated with any significant reduction in total Aβ or in Aβ40 or Aβ42 in any of the fractions analyzed (Fig. 3*a*). Although BAM-10 was selected on the basis of its ability to bind to and result in the disaggregation of diffuse Aβ deposits in Tg2576 mice when very high concentrations were applied directly to the brain, it is noteworthy that a similar effect on lowering Aβ in the brain was not apparent in mice receiving BAM-10 intraperitoneally.

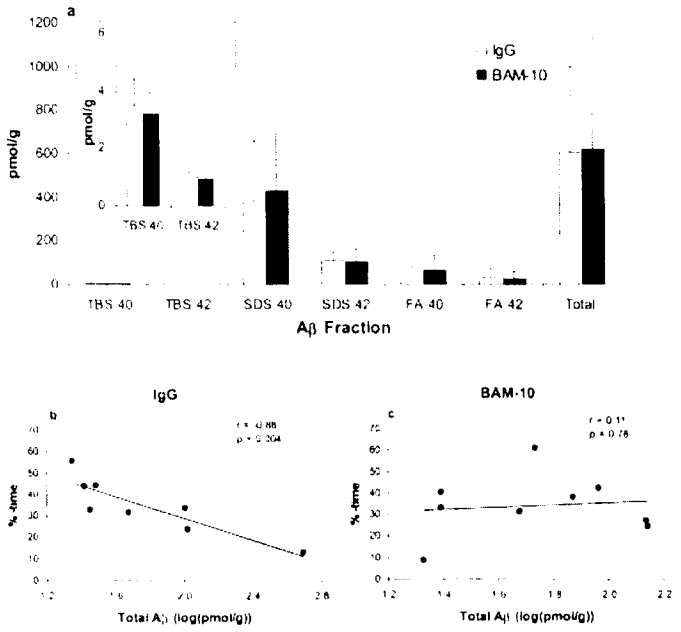

Figure 3. Aβ levels in Tg2576 mice treated with BAM-10 or nonspecific IgG antibody. Total Aβ is the sum of Aβ40 and Aβ42 in TBS, 2% SDS, and formic acid (FA) soluble fractions measured as described previously (Kawarabayashi et al., 2001). *a*, Treatment of mice with BAM-10 was not associated with a significant reduction in total Aβ or in Aβ40 or Aβ42 in any of the fractions analyzed (p values ranged from 0.2 to 0.9). Measurements represent means + SDs. Brain Aβ levels were correlated with memory in 8.7-month-old Tg2576 mice treated with BAM-10 or nonspecific IgG antibody. *b*, There was a significant inverse correlation between total Aβ and probe scores in control mice treated with nonspecific IgG. *c*, Treatment with BAM-10 eliminated the correlation between total Aβ and probe scores.

BAM-10 eliminates the inverse relationship between Aβ and memory

We have demonstrated previously that there was no obvious relationship between Aβ and memory in Tg2576 mice unless the mice were stratified by age, whereupon significant inverse correlations emerged (Westerman et al., 2002). Because Aβ rises very rapidly between 8 and 12 months of age (Kawarabayashi et al., 2001), tight stratification by age (in days) is necessary to obtain significant correlations between Aβ and memory during this time. To assess the effect of BAM-10 on the relationship between Aβ and memory in Tg2576 mice, we measured brain Aβ and post-treatment spatial reference memory in a second set of mice all exactly 8.7 months of age (all born within 1 d), treated with either BAM-10 or nonspecific IgG according to the same schedule as mice in the previously described longitudinal experiment using 9- to 11-month-old mice. As in the previous experiment, treatment with BAM-10 significantly improved memory but had no significant effect on total Aβ or on Aβ40 or Aβ42 in any of the three fractions (data not shown). Analysis of the first probe scores showed a significant negative correlation ($r = -0.88$; $p = 0.004$ by regression ANOVA) between total Aβ and memory in eight mice treated with nonspecific IgG (Fig. 3*b*). A similar negative correlation was observed in these mice for both Aβ40 and Aβ42 in each of the three fractions analyzed, with r values ranging from −0.70 to −0.87 and p values ranging from 0.005 to 0.05. Because the improved spatial learning and memory in mice treated with BAM-10 was not associated with a significant reduction in Aβ, the negative correlation between Aβ and probe scores was eliminated by BAM-10 treatment. As shown in Figure 3*c*, there was no significant correlation between Aβ and probe scores in nine mice treated with BAM-10 ($r = 0.11$; $p = 0.78$ by regression ANOVA).

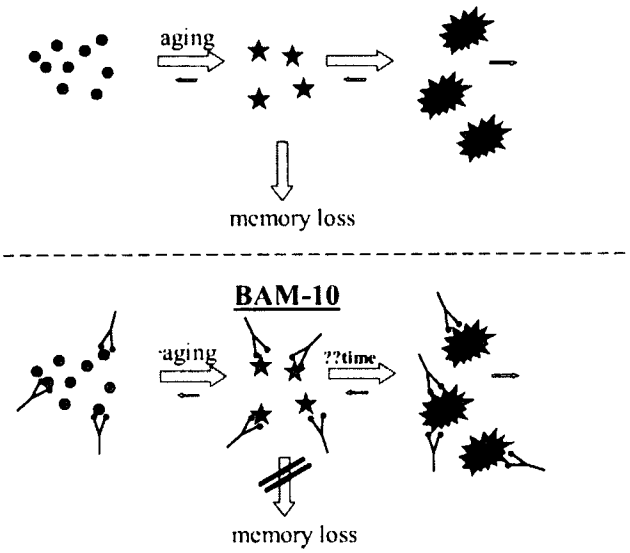

Figure 4. BAM-10 neutralizes the cognitively disruptive activity of small Aβ assemblies in the brain. *Top*, Memory loss in Tg2576 mice appears to be caused by small Aβ assemblies (*stars*) (Westerman et al., 2002) formed during the conversion of Aβ monomers (*circles*) to amyloid deposits (*starbursts*). Aging refers to the event or series of events occurring as animals age leading to the initial aggregation of monomeric Aβ. Little is known about what comprises these events. *Bottom*, BAM-10 penetrates into the brain, where it may bind to these small Aβ assemblies, neutralize their deleterious effects on cognitive function, and rapidly restore memory in Tg2576 mice. With prolonged treatment, a reduction in amyloid deposits may occur.

Although serum BAM-10 titers showed a 10-fold range in levels, there was no correlation between peripheral BAM-10 titers and probe scores ($r^2 = 0.0002$), indicating that the effect of BAM-10 on memory did not depend on blood levels of the antibody. These results suggest that BAM-10 enters the CNS and rapidly neutralizes the deleterious effects of small Aβ assemblies that interfere with cognitive function, thus restoring normal memory in Tg2576 mice.

DISCUSSION

Active immunization with Aβ was first shown to prevent amyloid deposition (Schenk et al., 1999) and was subsequently shown to prevent cognitive decline (Janus et al., 2000; Morgan et al., 2000) in two APP transgenic models of AD. Passive administration of Aβ antibodies intraperitoneally (Bard et al., 2000) as well as direct application of Aβ antibodies to the brain (Bacskai et al., 2001) resulted in a reduction of amyloid burden and Aβ levels and a rapid dispersal of deposits. None of these studies addressed the question of whether cognitive deficits, once present, could be restored to normal. Our results show that Aβ antibodies can indeed reverse behavioral deficits in a relatively short period of time. Although the IgG-treated mice performed nearly as well as BAM-10-treated mice after extensive training (data not shown), this does not diminish the observation that learning and memory occurred significantly more slowly in the IgG-treated group.

We postulate that BAM-10, like other Aβ antibodies (Bard et al., 2000), enters the CNS and acts by neutralizing soluble Aβ assemblies disrupting cognitive function. These results support the model we developed to explain the relationship between Aβ

and memory in Tg2576 mice (Westerman et al., 2002) (Fig. 4). Whether the same reversal effect would occur in older Tg2576 mice, where the presumably small amounts of BAM-10 entering the brain would bind to abundant amyloid deposits and therefore might be less available to neutralize soluble Aβ assemblies, is unknown. The direct interaction of BAM-10 with Aβ in the brain is in contrast to the mechanism of action proposed for m266 which, when chronically administered, lowers brain Aβ levels (DeMattos et al., 2001). It has been suggested that m266 exerts its action primarily from outside of the CNS, by creating a peripheral Aβ sink that draws Aβ out of the brain by mass action (DeMattos et al., 2001). We cannot exclude the possibility that BAM-10 exerts a similar indirect effect on brain Aβ in Tg2576 mice. Arguing against this mechanism, however, are the insignificant changes in brain Aβ after BAM-10 administration that are in contrast to the dramatic improvement in memory and the absence of any correlation between serum antibody titers and memory. Whether m266 and BAM-10 operate at distinct sites is an important question to resolve, because whether Aβ antibodies act within or outside of the CNS has important implications for potential inflammatory reactions in human Aβ immunization studies.

The rapid and full restoration of memory suggests that most if not all of the memory impairment in Tg2576 mice at this age occurs by this mechanism, and implies that little if any structural damage is associated with this type of Aβ-mediated brain dysfunction. The possibility that memory loss in humans might be reversed depends on the extent to which the same molecular mechanism that disrupts cognitive function in Tg2576 mice also exists in AD (Klein et al., 2001). Tg2576 mice may represent a model in which memory loss in certain early stages of AD can be studied. If Aβ species that functionally impair normal cognition contribute significantly to Alzheimer's dementia, especially in the early stages, then successfully targeting these species might improve or restore cognitive function.

Note added in proof. Rapid reversal of memory loss has also been shown in PDAPP mice receiving passively administered m266 Aβ antibodies (Dodart et al., 2002), which, together with the findings reported here, suggests a common mechanism for memory loss in transgenic APP mice.

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Effects of α-Synuclein Immunization in a Mouse Model of Parkinson's Disease

Eliezer Masliah,[1,2,*] Edward Rockenstein,[1] Anthony Adame,[1] Michael Alford,[1] Leslie Crews,[1] Makoto Hashimoto,[1] Peter Seubert,[3] Michael Lee,[3] Jason Goldstein,[3] Tamie Chilcote,[3] Dora Games,[3] and Dale Schenk[3]

[1]Department of Neurosciences
[2]Department of Pathology
University of California, San Diego
La Jolla, California 92093
[3]Elan Pharmaceuticals, Inc.
South San Francisco, California 94080

Summary

Abnormal folding of α-synuclein (α-syn) is thought to lead to neurodegeneration and the characteristic symptoms of Lewy body disease (LBD). Since previous studies suggest that immunization might be a potential therapy for Alzheimer's disease, we hypothesized that immunization with human (h)α-syn might have therapeutic effects in LBD. For this purpose, hα-syn transgenic (tg) mice were vaccinated with hα-syn. In mice that produced high relative affinity antibodies, there was decreased accumulation of aggregated hα-syn in neuronal cell bodies and synapses that was associated with reduced neurodegeneration. Furthermore, antibodies produced by immunized mice recognized abnormal hα-syn associated with the neuronal membrane and promoted the degradation of hα-syn aggregates, probably via lysosomal pathways. Similar effects were observed with an exogenously applied FITC-tagged hα-syn antibody. These results suggest that vaccination is effective in reducing neuronal accumulation of hα-syn aggregates and that further development of this approach might have a potential role in the treatment of LBD.

Introduction

Lewy body disease (LBD) is a heterogeneous group of disorders that includes Parkinson's disease (PD) and dementia with Lewy bodies (DLB) (Hansen and Galasko, 1992; Kosaka et al., 1984; McKeith, 2000), characterized by degeneration of the dopaminergic system (Shastry, 2001), motor alterations (Braak et al., 2002), cognitive impairment (Salmon et al., 1996), and formation of Lewy bodies (LBs) in cortical and subcortical regions (Trojanowski and Lee, 1998). The number of patients affected by these devastating conditions continues to climb as the population ages, creating a serious public health problem. The cause for LBD is controversial, and multiple factors probably play a role, including various neurotoxins and genetic susceptibility factors (Betarbet et al., 2000; Coleman et al., 1996; D'Amato et al., 1986; Forno et al., 1996; Jenner, 1998; Veldman et al., 1998).

*Correspondence: emasliah@ucsd.edu

In recent years, new hope for understanding the pathogenesis of this disease has emerged. Specifically, several studies have shown that the synaptic protein α-synuclein (α-syn) (Iwai et al., 1994) plays a central role in LBD pathogenesis since (1) this molecule accumulates in LBs (Spillantini et al., 1997; Takeda et al., 1998b; Wakabayashi et al., 1997), (2) mutations and multiplication in the α-syn gene are associated with rare familial forms of parkinsonism (Kruger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003), and (3) its expression in transgenic (tg) mice (Lee et al., 2002; Lee et al., 2004b; Masliah et al., 2000) and *Drosophila* (Feany and Bender, 2000) mimics several aspects of PD. Thus, the fact that accumulation of α-syn in the brain is associated with similar morphological and neurological alterations in species as diverse as humans, mice, and flies suggests that this molecule contributes to the development of LBD.

The mechanisms by which accumulation of α-syn leads to neurodegeneration and the characteristic symptoms of LBD are unclear. However, recent studies suggest that abnormal accumulation of α-syn oligomers in the synaptic terminals and axons plays an important role (Hashimoto and Masliah, 1999; Iwatsubo et al., 1996; Lansbury, 1999; Trojanowski et al., 1998).

Although experimental therapies utilizing neurotrophic factors (Kirik et al., 2004) and grafting of dopaminergic cells (Kim, 2004; Yoshizaki et al., 2004) have yielded promising results, alternative approaches directed at reducing the neuronal accumulation of α-syn are necessary. Viable strategies might include the use of viral vectors expressing antiaggregation molecules (Burton et al., 2003; Hashimoto et al., 2004) or factors such as immunotherapy, which promote the degradation or clearance of α-syn. For example, recent studies in a tg mouse model of Alzheimer's disease (AD) have shown that antibodies against β-amyloid 1-42 (Aβ) promote the removal of amyloid from the brain, resulting in improved cognitive performance (Janus et al., 2000; Morgan et al., 2000; Schenk et al., 1999). Moreover, a vaccination approach has been shown to be effective experimentally in tg mice at reducing the accumulation of prion protein (Sigurdsson et al., 2002) and huntingtin (Luthi-Carter, 2003; Miller et al., 2003), molecules that, like α-syn, accumulate intracellularly. In this context, we postulate that immunization of human (h)α-syn tg mice (a mouse model of LBD) with purified recombinant hα-syn might activate an immune response that will reduce hα-syn accumulation and, more importantly, neurotoxicity. This approach may be a suitable target for the development of an alternative immune therapy for PD and other disorders with LBs, parkinsonism, and dementia.

Results

Characterization of Antibody Titers, Relative Affinity, and Epitope Mapping

Three-month-old tg mice for group I and six-month-old tg mice for group II were immunized for 8 months with

Table 1. Summary of hα-Syn Antibody Titers and Relative Affinity, Corrected for Titer

Group	Antibody Relative Affinity by Miniblot	Antibody Affinity to Synapses	Antibody Affinity to Inclusions	Antibody Titers (First Bleed)	Antibody Titers (Second Bleed)	Antibody Titers (Third Bleed)
Group I/α-syn	109,147 ± 2700	1.9 ± 0.73	1.2 ± 0.4	2332 ± 500	2772 ± 1176	3644 ± 2365
Group I/CFA	113 ± 113	0.4 ± 0.1	0	19 ± 6.7	30 ± 12	7 ± 4
Group II/α-syn	235,747 ± 74,000	4.1 ± 0.9	2.8 ± 1.0	3813 ± 1200	2926 ± 976	1468 ± 641
Group II/CFA	400 ± 358	0.3 ± 0.2	0.1 ± 0.1	23 ± 9	21 ± 14	0.6 ± 0.6

recombinant hα-syn or Complete Freund's adjuvant (CFA) alone. Antibody titers in animals belonging to group I ranged from 200 to 20,000 (Table 1), and in mice from group II, titers ranged from 200 to 13,000 (Table 1). Levels of antibody relative affinity by immunoblotting were higher in mice from group II compared to immunized mice from group I (Table 1). By immunocytochemistry (ICC), sera from mice vaccinated with hα-syn showed either labeling of neurons (Figure 1A), intraneuronal inclusions (Figure 1B), or presynaptic terminals (Figure 1C) in hα-syn tg mice, but only displayed mild background staining in nontg mice (Figure 1D). Sera from mice treated with CFA alone showed nonspecific background staining in both hα-syn tg (Figure 1E) and nontg (Figure 1F) mice. By Western blot (WB) analysis, sera from mice vaccinated with hα-syn recognized hα-syn in tg mice (Figure 1G). In contrast, no immunoreactivity (IR) was observed with sera from mice treated with CFA alone (Figure 1H). Epitope mapping studies showed that in the vaccinated mice, antibodies recognized epitopes within the C terminus region of hα-syn, including amino acids (aa) 85–99, 109–123, 112–126, and 126–138.

Immunization Reduces hα-Syn Accumulation and Preserves Synaptic Density in tg Mice

To determine the effects of immunotherapy and antibody relative affinity on hα-syn accumulation, sections were analyzed by ICC with antibodies against hα-syn (Figure 2). Since antibody relative affinity (Figure 3A) and titers (Table 1) show a wide range of variability and linear regression analysis shows that antibody relative

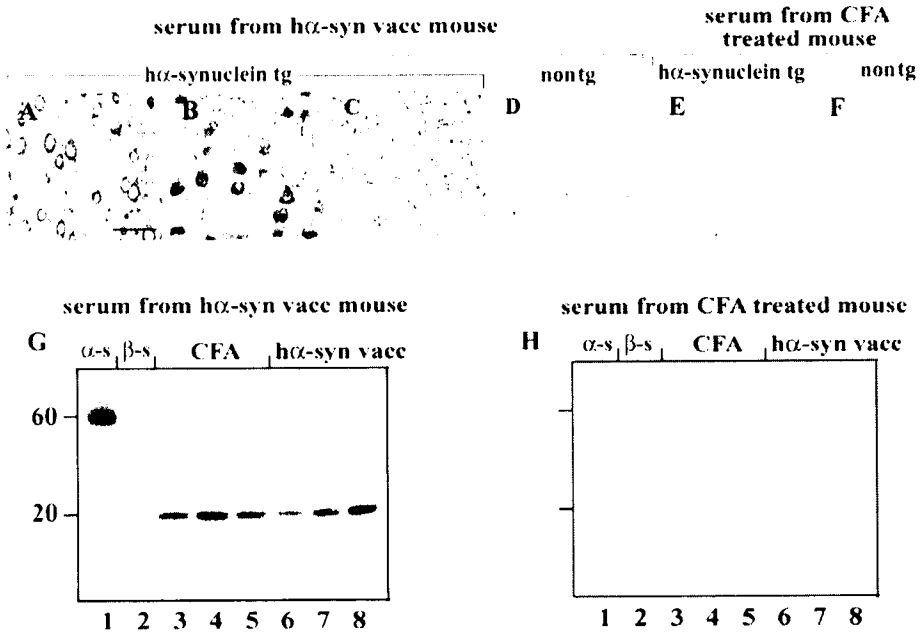

Figure 1. Immunocytochemical and Immunoblot Analysis with the Sera from Vaccinated Mice in the Brains of nontg and hα-Syn tg Mice

For panels (A)–(F), serial vibratome sections from nonimmunized hα-syn tg (Line D, 6-month-old) and nontg mice were immunostained with sera (normalized to 1:1000) from CFA and hα-syn-vaccinated (vacc) animals. All panels are from the temporal cortex. (A–C) Sections from hα-syn tg mice immunolabeled with the sera from immunized mice displayed neuronal staining (A), inclusion body staining (B), and granular neuropil immunolabeling suggestive of presynaptic terminals (C). (D) Section from a nontg mouse immunostained with the sera from immunized mice shows only mild background staining. (E and F) Sections from hα-syn tg (E) and nontg (F) mice immunolabeled with sera from CFA-treated mice showed only nonspecific background staining of the neuropil. Scale bar, 40 μm. For panels (G) and (H), homogenates from the brains of hα-syn tg mice treated with CFA or immunized with hα-syn were resolved by SDS-PAGE, and blots were probed with the sera from treated mice. (G) Immunoblot analysis with the sera from vaccinated mice shows a prominent band at approximately 19 kDa corresponding to recombinant hα-syn (lane 1, α-s), hα-syn in the brains of tg animals treated with CFA (lanes 3 through 5) or vaccinated with hα-syn (lanes 6 through 8). The sera did not recognize recombinant β-syn (lane 2, β-s). (H) Immunoblot analysis with the sera from CFA-treated animals shows no IR to recombinant hα-syn (lane 1, α-s), recombinant β-syn (lane 2, β-s) or in the brains of tg animals treated with CFA (lanes 3 through 5) or vaccinated with hα-syn (lanes 6 through 8).

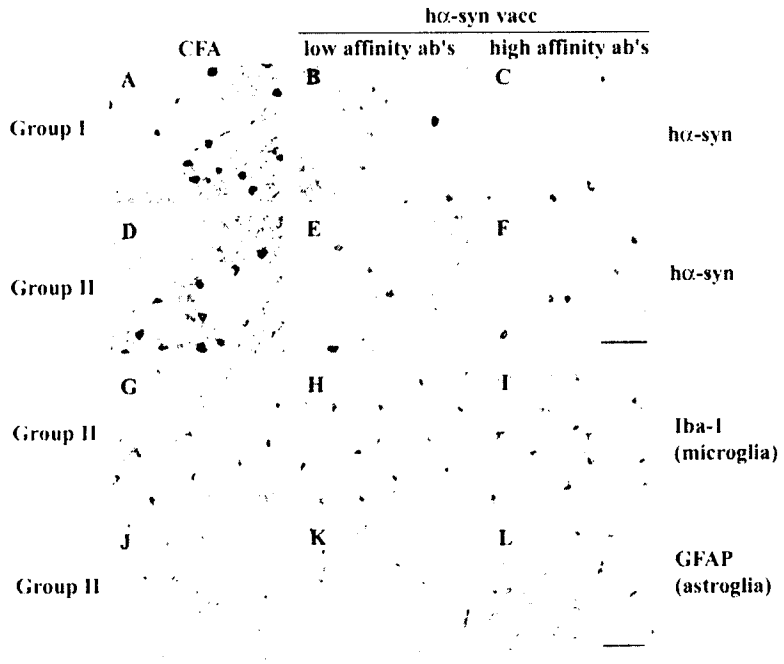

Figure 2. Patterns of hα-Syn IR and Glial Response in the Brains of Immunized hα-Syn tg Mice

Panels are from the temporal cortex of CFA or hα-syn-vaccinated mice reacted with antibodies against hα-syn, Iba1, or GFAP, developed with DAB, and analyzed by bright field microscopy. Panels (A)–(C) are representative images from the brains of group I mice (11-month-old), and panels (D)–(L) are from the brains of group II mice (14-month-old). Mice from both groups were divided into subsets of animals that produced antibodies with low (<100,000 units) or high (>100,000 units) relative affinities to hα-syn. (A and D) CFA-treated mice showed abundant hα-syn IR in intraneuronal inclusions as well as the neuropil. (B, E, C, and F) Immunized mice that produce low relative affinity (B and E) antibodies display a modest decrease in hα-syn-immunoreactive inclusions and neuropil labeling, and those that produce high relative affinity antibodies (C and F) demonstrate a more significant decrease in hα-syn-immunoreactive inclusions and neuropil labeling. (G–L) Compared to CFA-treated mice (G and J), animals vaccinated with hα-syn show only a mild increase in microglial IR (Iba-1, [H and I]) and mild astroglial activation (GFAP, [K and L]). Scale bar, 50 μm.

affinity was one of the strongest predictors for the effects on reducing the neuropathology on the vaccinated hα-syn tg mice (Table 2), animals were divided into subgroups consisting of those that show low (<100,000 units; Figures 2B, 2E, 2H, and 2K) and high (>100,000 units; Figures 2C, 2F, 2I, 2L, and 3A) relative affinity to hα-syn (expressed as units of intensity volume). In tg mice treated with CFA alone, abundant hα-syn IR was observed in the neuropil as well as in intraneuronal inclusions (Figures 2A and 2D). In contrast, immunized mice from both groups showed a reduction in the number of inclusions in the temporal cortex (Figure 3B). Compared to mice producing low relative affinity antibodies (Figures 2B and 2E), this effect was more pronounced in mice producing antibodies with high relative affinity to hα-syn (Figures 2C and 2F). Moreover, immunization resulted in a decrease in hα-syn IR in the neuropil of tg mice producing antibodies with high relative affinity to hα-syn (Figures 2C and 2F), with a greater effect in mice from group II (Figure 2F) than group I (Figures 2C and 3D), compared to CFA-treated tg mice (Figures 2A and 2D). To investigate whether the effects of the immunization were accompanied by a neuroinflammatory response, immunocytochemical analysis with antibodies against the microglial marker (Iba-1) and the astroglial marker (glial fibrillary acidic protein [GFAP]) were performed. These studies showed that in both CFA-treated (Figures 2G and 2J) and hα-syn-vaccinated (Figures 2H, 2I, 2K, and 2L) animals, there was a mild increase in Iba-1 and GFAP IR; however, no overt differences were detected between the groups.

To further investigate the effects of the vaccination on the synapses, higher-resolution analysis with the laser scanning confocal microscope (LSCM) was performed. These studies confirmed that compared to CFA controls (Figures 3D, 4A, and 4C) the reduction in the neuropil immunostaining was associated with decreased accumulation of hα-syn in presynaptic terminals (Figures 3D, 4B, and 4D). To ascertain the effects of the immunotherapy on neuropil integrity, sections were immunostained with an antibody against synaptophysin (Figures 4E–4H). Compared to nontg mice, hα-syn tg mice treated with CFA alone (Figures 4E and 4G) showed an average of 20% decrease in the number of synaptophysin-immunolabeled terminals (Figure 3C). In contrast, immunized mice from both groups I and II showed levels of synaptophysin IR comparable to nontg controls (Figures 3C, 4F, and 4H). This effect was more prominent in mice that produced antibodies with high relative affinity to hα-syn (Figure 3C).

To rule out immunization masking effects, control experiments were performed by comparing the levels of murine (m)α-syn. Similar to hα-syn, mα-syn IR was abundant in the neuropil in association with nerve terminals, but was absent from the neuronal cell bodies and in the inclusions (Figures 4I–4L). Both in CFA (Figures 4I and 4K) and hα-syn immunized mice (Figures 4J and 4L), patterns and levels of mα-syn were comparable. Additional analysis of the immunization specificity was performed by comparing levels of β-syn IR, a close α-syn homolog (Iwai et al., 1994). Abundant β-syn IR was observed in the neuropil in association with the presynaptic terminals, and mild immunolabeling was detected in the neuronal cell bodies, but not in the inclusions (Figures 4M–4P). Compared to tg mice treated with CFA alone (Figures 4M and 4O), no differences in the patterns and levels of β-syn were found in mice immunized with hα-syn (Figures 4N and 4P). These studies suggest that vaccination specifically affects hα-syn but not other related synaptic molecules.

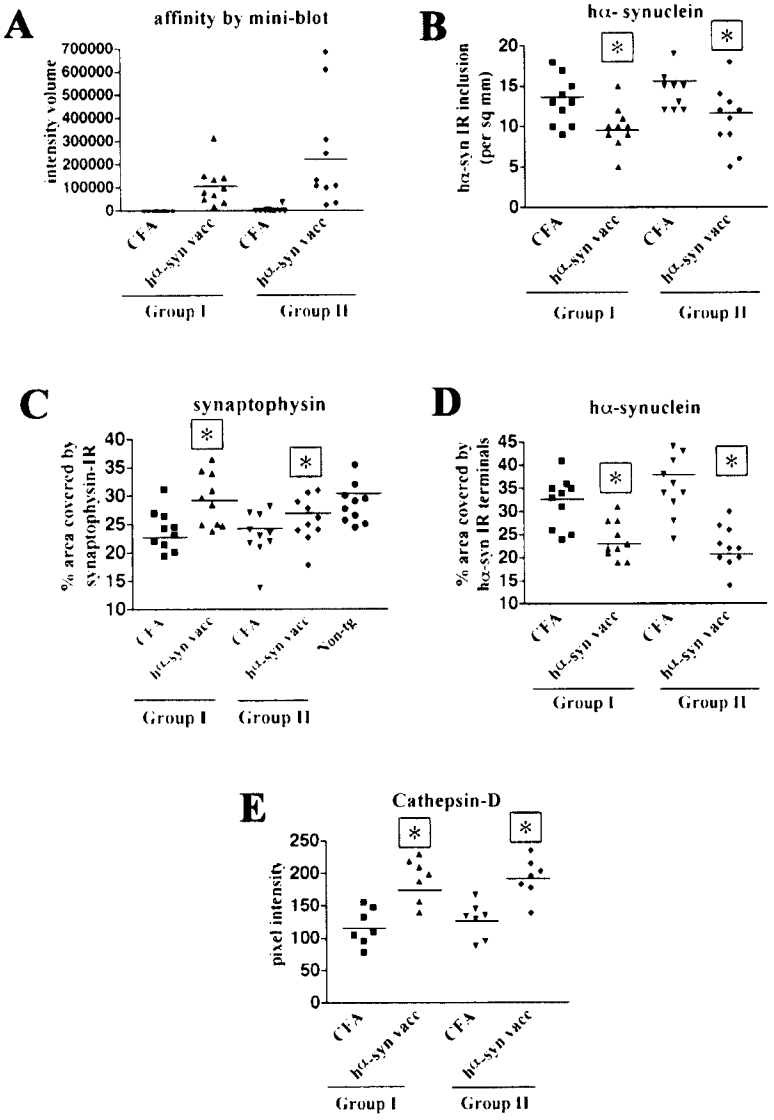

Figure 3. Image Analysis of the Levels of hα-Syn IR and Other Markers of Neurodegeneration

(A) Relative affinity by mini-blot of hα-syn antibodies produced by mice treated with CFA alone or immunized with hα-syn was assessed. (B) Scatterplot of number of hα-syn-positive inclusions in the temporal cortex of mice treated with CFA alone or immunized with hα-syn. Vaccination resulted in a significant decrease in the number of inclusions in both groups compared to controls, an effect more pronounced in group II mice (14-month-old) as opposed to group I (11-month-old). (C) Percent area of the neuropil occupied by synaptophysin-immunoreactive terminals in the temporal cortex. Compared to nontg controls, in tg mice treated with CFA alone, the number of synaptophysin-immunolabeled terminals decreased by an average of 20%. In contrast, tg mice from both groups showed levels of synaptophysin IR comparable to nontg controls. (D) Percent area of the neuropil occupied by hα-syn-immunoreactive terminals in the temporal cortex. In tg mice from both groups vaccinated with hα-syn, there was a decrease in the accumulation of hα-syn in synaptophysin-immunoreactive terminals. (E) Levels of cathepsin D IR (measured as pixel intensity) in CFA-treated and hα-syn-vaccinated tg mice. In vaccinated tg mice, there were significantly higher levels of cathepsin D IR in both groups compared to CFA controls. * = significant difference compared to CFA-treated hα-syn tg mice ($p < 0.05$, unpaired, two-tailed Student's t test).

To better characterize the effects of vaccination on hα-syn aggregation in the synapses, double immunocytochemical analysis with antibodies against hα-syn and the presynaptic terminal marker synaptophysin and WB analysis with synaptosomal preparations were performed. Under physiological conditions, hα-syn is localized primarily to the presynaptic boutons (Iwai et al., 1994), and in LBD and the tg mice, increased accumulation of hα-syn in the synapses is associated with functional deficits and synapse loss (Hashimoto et al., 2001). Confocal imaging showed that in comparison to CFA-treated hα-syn tg mice (Figure 3D), vaccinated mice displayed a decrease in the proportion of synaptophysin-immunoreactive nerve terminals in the neocortex that show hα-syn immunolabeling (Figure 3D). Immunoblot analysis with synaptosomal preparations (Figure 5A, upper panel) showed that immunization decreased the accumulation of hα-syn higher molecular weight bands (Figure 5A, upper panel, and Figure 5B), but no effects were observed on mα-syn (data not shown). Furthermore, compared to CFA-treated tg mice, levels of synaptophysin IR were higher in the synaptosomal preparations from immunized mice (Figure 5A, lower panel, and Figure 5B). These results suggest that immunotherapy might ameliorate the neuronal damage in the brains of tg mice by reducing the accumulation of potentially toxic hα-syn in the synapses.

Immunization Effects Are Dependent on the Relative Affinity of Antibodies to Recognize Membrane-Associated Aggregated hα-Syn

Since abnormal accumulation of hα-syn is associated with translocation of hα-syn from the cytosol to the membrane, it is possible that this might explain the antibodies' ability to recognize abnormally accumulated hα-syn in the immunized tg mice. WB analysis showed abundant monomeric hα-syn in both the cytosol (Figure 5C) and membrane (Figure 5D). However, oligomeric hα-syn was more abundant in the membrane fractions where, compared to CFA controls, immunization with hα-syn resulted in decreased accumulation of hα-syn oligomers (Figure 5D).

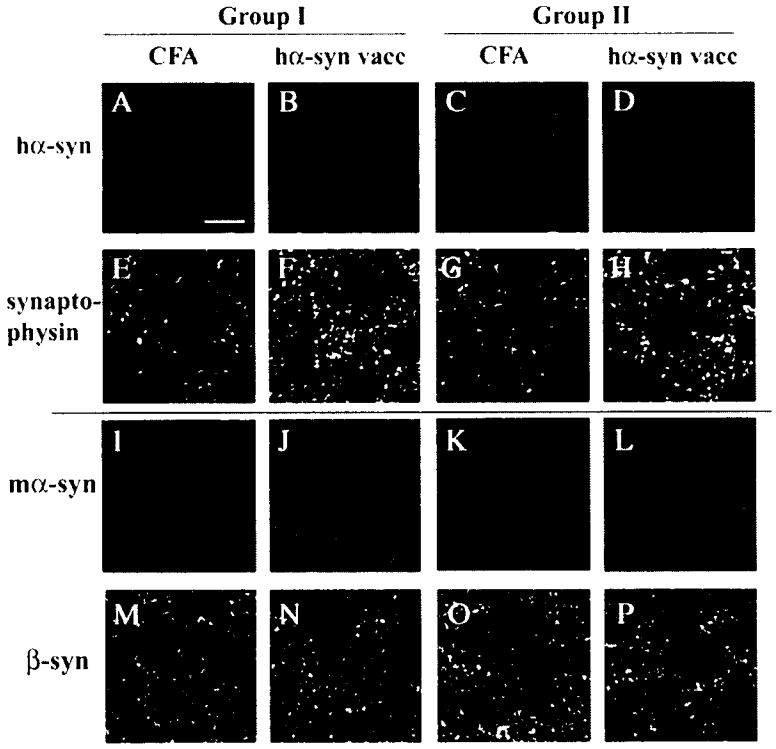

Figure 4. Immunocytochemical Analysis of hα-Syn, Synaptophysin, mα-Syn, and β-Syn IR in the Brains of hα-Syn tg Mice from Groups I and II

All panels are from the temporal cortex of CFA-treated or vaccinated mice. Group I, 11-month-old; group II, 14-month-old. (A–D) Sections were reacted with an antibody against hα-syn, labeled with Tyramide Red and imaged with the LSCM. Compared to tg mice treated with CFA alone (A and C), immunized mice (B and D) showed a reduction in the number of inclusions in the temporal cortex as well as a decrease in hα-syn IR (presynaptic terminals) in the neuropil. (E–H) Sections were immunostained with an antibody against synaptophysin and FITC-labeled for imaging with the LSCM. In tg mice treated with CFA alone (E and G), levels of synaptophysin IR were reduced, whereas immunized mice showed levels of synaptophysin IR comparable to nontg controls. (I–P) Sections were reacted with an antibody against mα-syn and labeled with Tyramide Red (I–L) or with an antibody against and β-syn and FITC-labeled (M–P), and imaged with the LSCM. (I–L) Levels of endogenous mα-syn remain unchanged in tg mice treated with CFA alone (I and K) and immunized mice (J and L). All display mα-syn IR in the neuropil, but there is no labeling of the intraneuronal inclusions as is found with antibodies against hα-syn. (M–P) Both hα-syn tg mice treated with CFA alone (M and O) and tg mice vaccinated with hα-syn (N and P) display abundant β-syn IR in the neuropil and mild immunolabeling in the neuronal cell bodies, but there is no labeling of the intraneuronal inclusions as is found with antibodies against hα-syn. Scale bar, 10 μm.

To better understand which factors might predict the effectiveness of the immunotherapy, linear regression analysis was performed between the neuropathological markers of hα-syn accumulation and the antibody titers and relative affinity (Table 2). A significant correlation was observed between relative antibody affinity by immunoblot and levels of hα-syn IR in the synapses, but not with the numbers of neuronal inclusions. Similarly, relative antibody affinity to recognize synapses by ICC was inversely correlated with levels of hα-syn in the synapses and directly correlated with the percent area occupied by synaptophysin-labeled nerve terminals, but not with the numbers of neuronal inclusions. Antibody titers correlated with the percent area of the neuropil labeled with the anti-hα-syn antibody, but negatively correlated with neuronal inclusions (Table 2). These results suggest that the relative immunoblot reactivity of the anti-hα-syn antibodies and to some extent the antibodies' titers correlate with the reduction of neuronal hα-syn accumulation.

Anti-hα-Syn Antibodies Are Internalized and Trigger Clearance of hα-Syn Aggregates via Lysosomal Activation

To determine if anti-hα-syn antibodies generated by the immunized mice recognize hα-syn aggregates in the tg mice, immunocytochemical analysis was performed with horse anti-mouse IgG antibodies. Double immunostaining experiments showed that compared to CFA-treated tg mice (Figures 6A–6C), in hα-syn vaccinated mice, the anti-mouse IgG and the hα-syn IR were colocalized in the periphery of the cell bodies (Figures 6D–6F), the neuritic processes and synapses (Figures 6G–6I). In hα-syn containing neurons, the two markers were detected in granular subcellular structures averaging 0.4–0.8 μm in diameter (Figures 6G–6L). Furthermore, these granular structures displayed cathepsin D IR, suggesting that the internalized anti-hα-syn antibodies reacted with hα-syn within lysosomes (Figures 6M–6O).

To corroborate that vaccination resulted in lysosomal activation, cathepsin D IR was analyzed. Compared to

Table 2. Summary of Correlations between Antibody Relative Affinity, Neuropathology, and Titers

Neuropathological Markers	Antibody Relative Affinity by Miniblot	Antibody Affinity to Synapses	Antibody Affinity to Inclusions	Antibody Affinity to Neurons	Antibody Titers (First Bleed)
Number of α-syn (+) inclusions	−0.11	0.04	0.12	−0.21	0.1
% area of neuropil α-syn (+) synapses	−0.46 (p = 0.003)	−0.41 (p = 0.009)	−0.43 (p = 0.005)	0.06	−0.47 (p = 0.007)
% area of neuropil synaptophysin (+) synapses	0.06	0.35 (p = 0.04)	0.01	0.04	0.12
Antibody relative affinity by miniblot	—	0.74 (p = 0.0001)	0.70 (p = 0.0001)	−0.16	0.85 (p = 0.0001)
Antibody titers (first bleed)	0.85 (p = 0.0001)	0.62 (p = 0.0001)	−0.18	0.81 (p = 0.0001)	—

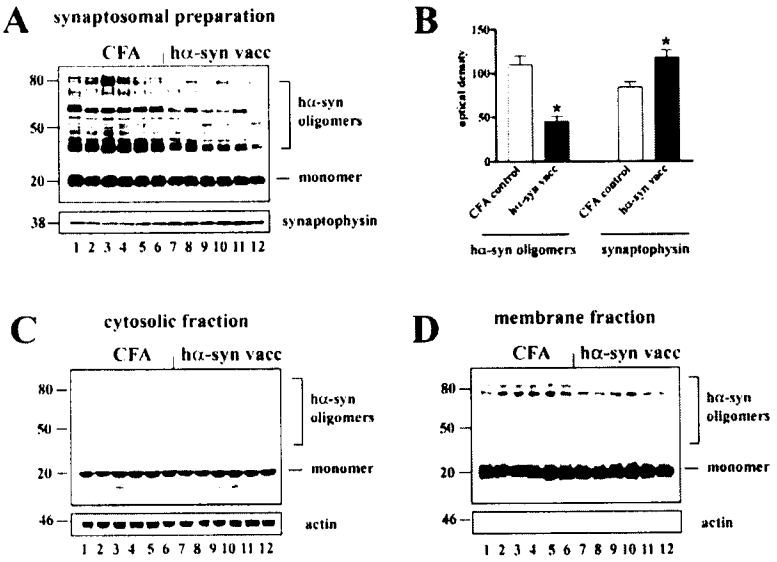

Figure 5. WB Analysis of the Levels of hα-Syn IR in Synaptosomal, Membrane, and Cytosolic Preparations from Vaccinated Animals

(A and B) Compared to brains of tg mice treated with CFA alone (lanes 1 through 6), in hα-syn-vaccinated tg mice (lanes 7 through 12), levels of hα-syn oligomers were decreased (upper panel), while levels of synaptophysin IR increased in the immunized animals (lower panel). (C and D) Compared to the cytosolic fraction of the brains of tg mice (C), a majority of hα-syn high molecular weight oligomers are present in the membrane fraction (D), where compared to mice treated with CFA alone (lanes 1 through 6), there is a decrease in hα-syn oligomers in the fractions from vaccinated animals (lanes 7 through 12). * = significant difference compared to hα-syn tg mice treated with CFA alone ($p < 0.05$, unpaired, two-tailed Student's t test). Animals were 14 months old.

CFA controls (Figures 7A–7C), in vaccinated mice there was increased cathepsin D IR (Figures 3E and 7D–7F). Similar increases in levels of cathepsin D IR were detected in the hα-syn-vaccinated mice from both groups when compared to the CFA-treated group (Figure 3E). In vaccinated tg mice, the cathepsin D-immunolabeled lysosomes were colocalized with discrete hα-syn-immunoreactive granular aggregates (Figure 7F), but no colocalization was observed in CFA-treated tg mice (Figure 7C).

To determine if exogenously applied antibodies recognize hα-syn aggregates in tg mice and activate lysosomal pathways, purified monoclonal anti-hα-syn antibodies were tagged with fluorescein isothiocyanate (FITC) and injected into tg and nontg animals. The FITC-tagged anti-hα-syn recognized hα-syn aggregates in the neuronal cell bodies and synapses of tg animals (Figure 7G–I), but not the endogenous mα-syn in the nontg controls (Figures 7J–7L). Furthermore, in tg mice treated with the FITC-tagged antibody, there was an increase in neuronal cathepsin D IR and colocalization with hα-syn (Figures 7M–7O) compared to nontg controls (Figures 7P–7R). No specific labeling of hα-syn or increase in cathepsin D IR was detected in control experiments where tg and nontg mice were treated with a nonimmune FITC-tagged IgG (data not shown). These studies suggest that circulating antibodies might recognize abnormally aggregated hα-syn associated with the neuronal membrane, which in turn might lead to clearance via lysosomal activation.

Discussion

The present study showed that hα-syn antibodies generated in vaccinated mice reduced the abnormal accumulation of this protein in the neuronal cell bodies and synapses and ameliorated the loss of synaptophysin-immunoreactive nerve terminals in hα-syn tg mice. This is consistent with recent in vitro studies showing that intracellular antibodies can inhibit α-syn aggregation (Emadi et al., 2004; Zhou et al., 2004). Moreover, our findings are consistent with studies showing that immunotherapy might reduce the accumulation of extracellularly deposited proteins such as Aβ (Games et al., 2000; Lemere et al., 2003; Morgan et al., 2000) and of intracellular proteins such as huntingtin (Luthi-Carter, 2003), as well as membrane-associated molecules such as prion proteins (Bainbridge and Walker, 2003; White et al., 2003; White and Hawke, 2003). More recently, a study showed that immunotherapy with copolymer-1-immune cells might reduce neurodegeneration in the MPTP model of PD (Benner et al., 2004). While this immunotherapy approach focused on protecting via a general anti-inflammatory mechanism, the present study sought to elicit a specific response to promote degradation of toxic hα-syn. The mechanisms through which the antibodies generated in the vaccinated mice might recognize and promote the clearance of intracellular hα-syn aggregates and other neuronal proteins are less clear. One possibility is that circulating antibodies in the CNS might recognize and cross-link abnormally conformed proteins in the neuronal cell surface (Figure 8). For this, at least a limited amount of the target antigen must be present in the neuronal cell membrane (Luthi-Carter, 2003). A similar mechanism has been described in the antibody-mediated clearance of viruses from cells (Garzon et al., 1999; Ubol et al., 1995). Interestingly, in the case of α-syn, under basal conditions the monomeric forms of this molecule are almost exclusively present in the cytosolic fraction (Eliezer et al., 2001; Iwai, 2000). In contrast, under pathological circumstances leading to α-syn aggregation, oligomers and protofibrils can be found in the plasma membrane (Eliezer et al., 2001; Lansbury, 1999; Dixon et al., 2005), where they could potentially be recognized by circulating antibodies. Supporting this possibility, immunoblot and immunocytochemical analysis showed that vaccination reduced hα-syn accumulation in the membrane and that mouse IgG and hα-syn were colocalized in the periphery of the neurons, suggesting

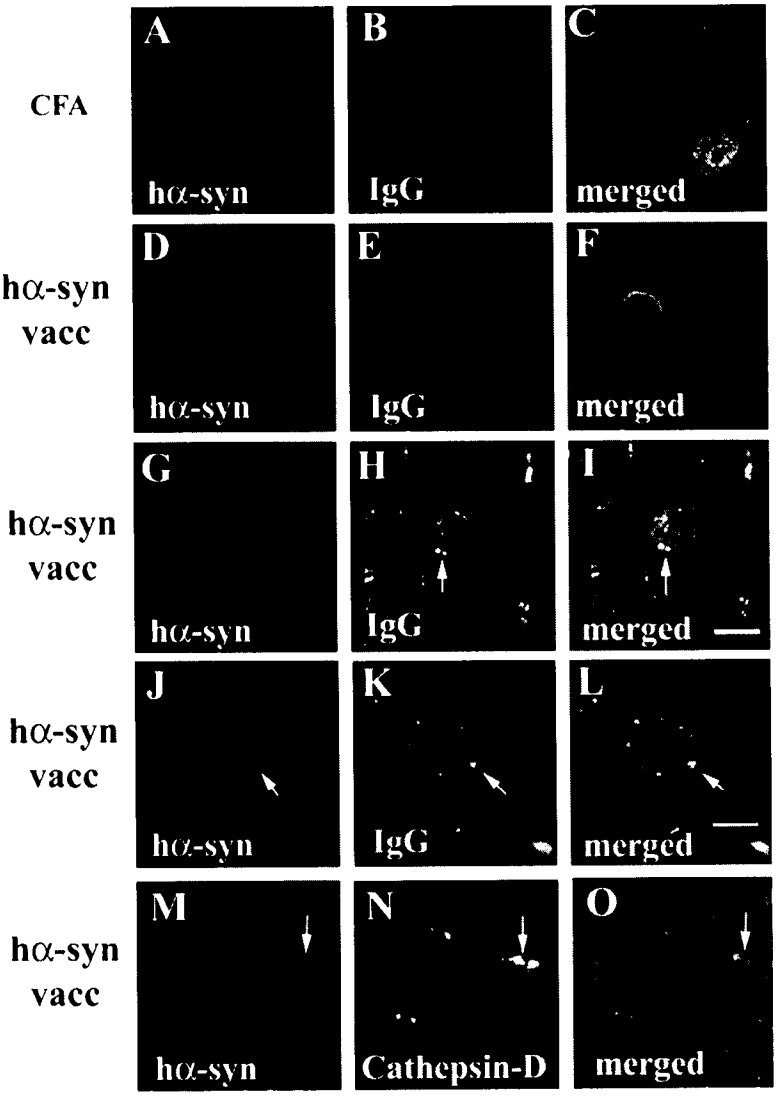

Figure 6. Double Immunocytochemical Analysis of the Patterns of hα-Syn and anti-IgG or Cathepsin D IR in Vaccinated Animals

All images are from the temporal cortex. Sections from CFA-treated and hα-syn vaccinated animals from group II were double labeled with antibodies against hα-syn (panels to the left, red) and FITC-tagged anti-mouse IgG or cathepsin D (central panels, green) and imaged with the LSCM. The images to the right represent the merged panels (yellow indicates colocalization of the two markers), demonstrating that neuronal cell bodies labeled by the anti-mouse IgG and the intraneuronal granular structures labeled by cathepsin D displayed hα-syn IR (arrows). (A–C) Tg mice treated with CFA show characteristic inclusions that react strongly with hα-syn antibodies and show background levels of reactivity with anti-mouse IgG. (D–F) Tg mice vaccinated (vacc) with hα-syn show a close colocalization between hα-syn and anti-mouse IgG in the periphery of the neuronal cell bodies. (G–L) Additionally, the anti-mouse IgG immunostaining is colocalized with hα-syn in small granular structures within the cell bodies (arrows). (M–O) These small granular structures also displayed lysosomal cathepsin D IR that colocalized with hα-syn immunolabeling. Scale bar, 10 μm (A–I), 5 μm (J–L). Animals were 14 months old.

that such interactions might take place in the neuronal surface.

Since circulating antibodies might be able to recognize membrane bound α-syn, there are several possibilities as to how they might promote the clearance of intracellular aggregates. Antibodies in close opposition with the neuronal surface might enter the neurons alone via surface receptors or in association with membrane bound α-syn and promote lysosomal degradation (Figure 8). This process may be mediated by receptor-dependent or receptor-independent endocytosis of the antibody or antibody-antigen complex, followed by fusion with lysosomes and incorporation of α-syn aggregates (Figure 8). Supporting this possibility, anti-mouse IgG, an exogenously applied FITC-tagged anti-hα-syn antibody, and cathepsin D colocalized with hα-syn aggregates. Consistent with the possibility that internalization of the antibody complex might play a role in clearance of α-syn aggregates, previous studies have shown that neurons can uptake immunoglobulins (Fabian and Petroff, 1987) and internalize some through the Thy 1.1 receptor in the neuronal and synaptic plasma membrane (Fabian, 1990). Other receptors that might be involved in this process and mediate the endocytosis of antibodies or antibody-antigen complexes include lipoprotein receptor-related protein (LRP) (Herz et al., 1990; Kounnas et al., 1995). This receptor is highly abundant in neurons (Schneider and Nimpf, 2003; Hussain, 2001), and macromolecules endocytosed via LRP target the lysosomal pathway (Gonias et al., 2004), suggesting that clearance of intracellular hα-syn aggregates might involve lysosomal degradation. In support of this mechanism, the present study showed that in the neurons of vaccinated mice, granular hα-syn-immunoreactive structures were also labeled with antibodies against the lysosomal marker cathepsin D. Similarly, previous studies have shown that immunoglobulins internalized by neurons form granular cytoplasmic structures that display lysosomal-like activity (Meeker et al., 1987). Moreover, recent studies have shown that lysosomally dependent autophagy might facilitate the clearance of α-syn oligomers (but not fibrils) and there-

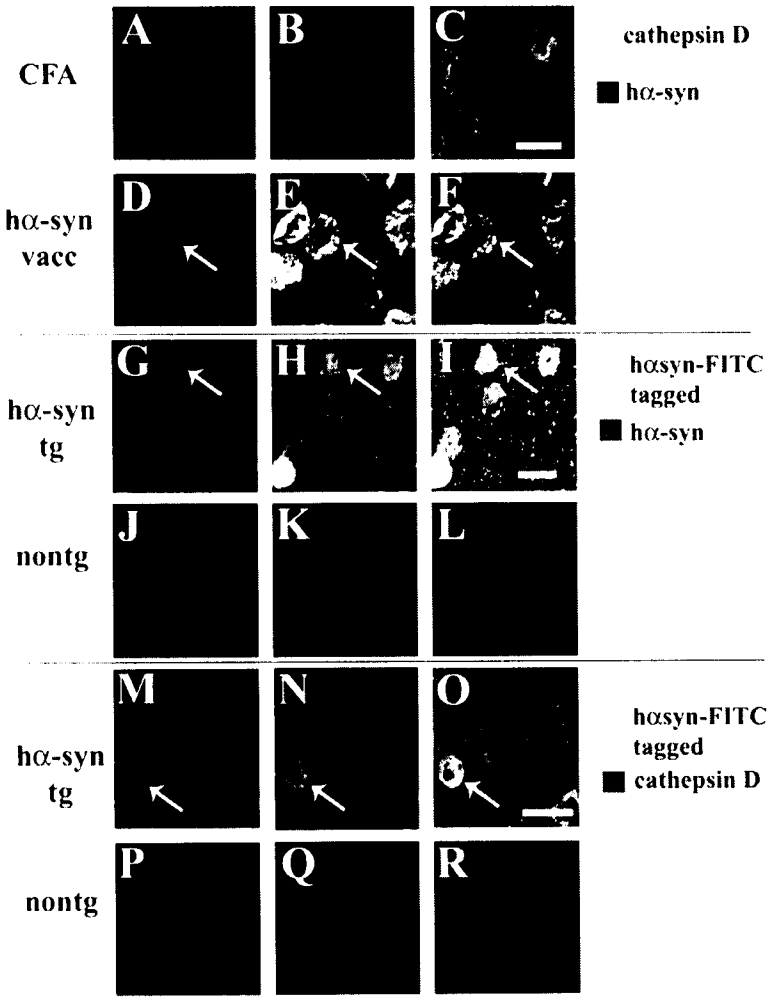

Figure 7. Antibody Recognition of hα-Syn and Lysosomal Activation in Vaccinated hα-Syn tg Animals

All panels are LSCM images from the temporal cortex. Panels (A)–(F) are from CFA-treated or immunized hα-syn tg mice from group II (14-month-old), and panels (G)–(R) are from non-immunized nontg and hα-syn tg animals (5-month-old) that received intracerebral injections of an FITC-tagged hα-syn antibody. (A–C) Images from sections of a hα-syn tg mouse treated with CFA alone double immunolabeled with antibodies against hα-syn (red) and the lysosomal marker cathepsin D (green). (D–F) Images from sections of a vaccinated hα-syn tg mouse double immunostained with antibodies against hα-syn (red) and cathepsin D (green). Yellow indicates colocalization between granular hα-syn-immunoreactive structures and cathepsin D (arrows). Note the higher levels of cathepsin D IR in these images. (G–I) Images from sections of a hα-syn tg mouse that received intracerebral injections of an FITC-tagged antibody against hα-syn (green) coimmunolabeled with a different antibody against hα-syn (red). Yellow indicates colocalization between injected and immunolabeled anti-hα-syn antibodies. (J–L) Images from sections of a nontg mouse that received intracerebral injections of an FITC-tagged antibody against hα-syn (green) coimmunolabeled with a different antibody against hα-syn (red). (M–O) Images from sections of a hα-syn tg mouse that received intracerebral injections of an FITC-tagged antibody against hα-syn (green) coimmunolabeled with an antibody against cathepsin D (red). Yellow indicates colocalization between hα-syn and cathepsin D (arrows). (P–R) Images from sections of a nontg mouse that received intracerebral injections of an FITC-tagged antibody against hα-syn (green) coimmunolabeled with an antibody against cathepsin D (red). Scale bar, 15 μm.

fore may play a protective role (Cuervo, 2004; Cuervo et al., 2004; Lee et al., 2004a). These studies suggest that in the immunized mice, internalized antibodies might promote degradation of hα-syn aggregates via activation of lysosomal pathways. Alternatively, active vaccination might also promote the clearance of α-syn aggregates via an immune-mediated cellular response. This is unlikely, because in the present study, no apparent infiltration by lymphocytes was noted and only mild microglial activation was detected. However, it is important to consider this possibility because active immunization in other neurodegenerative disorders has been associated in some cases with vasculitis and autoimmune response (Ferrer et al., 2004), and caution should be exercised when considering the use of active immunization as a potential therapy in patients with PD. For this reason we are currently investigating the effects of passive immunization with antibodies against various regions of α-syn.

Antibodies against α-syn not only decreased accumulation in neuronal cell bodies, but also in neuropil, suggesting that the immunotherapy also reduced the accumulation of hα-syn aggregates in the synapses. This effect was associated with amelioration of the neurodegenerative pathology. It is unclear whether this might be accompanied by the improvement of behavioral deficits; experiments are currently underway to assess this possibility. The ability of the antibodies to recognize neuronal cell bodies and synapses was a good predictor of their ability to reduce hα-syn aggregation and neurodegeneration at least as determined by synaptic integrity. This is consistent with studies in AD, where antibodies with high affinity for plaques displayed a better response (Hock et al., 2002). While for Aβ, the most effective antibodies recognize the N terminus (Bard et al., 2003), for hα-syn, high relative affinity antibodies recognized epitopes within the C terminus. Recent studies have shown that C-terminal fragments of α-syn assemble into protofibrils and associate with the membrane (McLean et al., 2000; Kim et al., 2002; McLean et al., 2000). Deletion of key amino acids 125–140 within the C-terminal domain greatly alters α-syn aggregation (Kim et al., 2002), and in the brains of patients with LBD as well as in tg animal models, there is abundant accumulation of C-terminal α-syn fragments (Iwatsubo et al., 1996; Takeda et al., 2000; Takeda et al., 1998a). These studies suggest that the antibodies' ability to recognize the C-terminal region might be important for the therapeutic effects.

Considerable effort has recently been directed to-

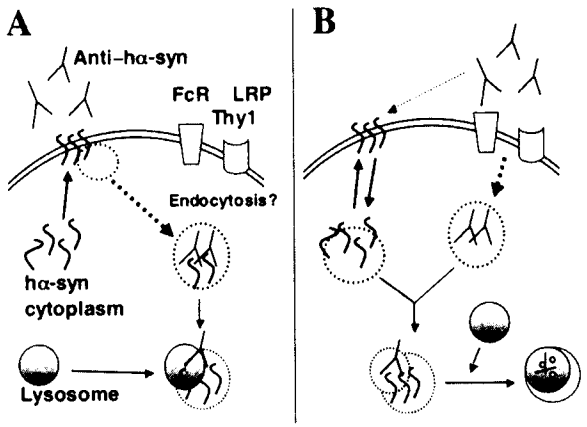

Figure 8. Potential Mechanisms of Antibody-hα-Syn Recognition and Targeting to the Lysosomal Pathway

(A) Antibodies might recognize hα-syn bound to the membrane. This complex might in turn be endocytosed and targeted for lysosomal degradation. (B) Receptor-mediated endocytosis of the antibodies and hα-syn aggregates that in turn could be targeted for lysosomal degradation.

ward the development of immunization therapies for neurodegenerative disorders, including the use of active and passive immunization strategies (Frenkel and Solomon, 2001; Morgan et al., 2000; Schenk et al., 1999), DNA vaccination (Luthi-Carter, 2003), and, more recently, adoptive transfer of immune cells to protect nigrostriatal neurons (Benner et al., 2004). Adding to this growing body of evidence supporting a role of immunization in the management of neurodegenerative disorders, the present study suggests that vaccination is effective in reducing the neuronal accumulation of toxic hα-syn aggregates and that further development of this approach might have a potential place in the treatment of LBD.

Experimental Procedures

Vaccination of hα-Syn tg Mice

For this study, heterozygous tg mice (Line D) expressing hα-syn under the regulatory control of the platelet-derived growth factor-β (PDGFβ) promoter (Masliah et al., 2000) were used. These animals were selected because they display abnormal accumulation of detergent-insoluble hα-syn and develop hα-syn-immunoreactive inclusion-like structures in the brain. Although some nuclear staining has been observed in this model, distinct cytoplasmic inclusion-like structures have been consistently identified by confocal and electron microscopy (Masliah et al., 2000; Masliah et al., 2001; Rockenstein et al., 2002). Furthermore, these animals also display neurodegenerative and motor deficits that mimic certain aspects of LBD. Experimental animals were divided into two groups. For the first group, a total of 20 young (3-month-old) tg mice were immunized for 8 months with purified recombinant hα-syn expressed in *E. coli* from sequence-verified hα-syn cDNA (n = 10) or CFA alone (n = 10). For the second group, a total of 20 young adult (6-month-old) tg mice were immunized for 8 months with recombinant hα-syn (n = 10) or CFA alone (n = 10). The immunization protocol consisted first of an injection with recombinant hα-syn (80 μg/ml; 100 μl) with CFA (Sigma-Aldrich, St. Louis, MO). Two weeks later, mice received another injection of hα-syn (80 μg/ml; 100 μl) with incomplete Freund's Adjuvant (FA), followed by reinjection once a month (for the subsequent 7 months) with hα-syn (80 μg/ml; 100 μl) in phosphate-buffered saline. Recombinant hα-syn was prepared and purified and tested for endotoxins at Elan following a modified version of a previously described protocol (Hashimoto et al., 1998).

Determination of Antibody Titers and Relative Affinity to hα-Syn

Antibody levels in plasma were determined using 96-well microtiter plates coated with 0.4 μg per well of purified full-length hα-syn. Samples were incubated overnight followed by goat anti-mouse IgG alkaline phosphatase-conjugated antibody (1:7500, Promega, Madison, WI). The plate was read at wavelengths of 450 nm and 550 nm. Results were plotted on a semi-log graph with relative fluorescence units versus serum dilution. Antibody titer was defined as the dilution at which there was a 50% reduction from the maximal antibody binding.

To determine the relative affinity of the antibodies for hα-syn, three assays were performed. In the first, brain homogenates from nonimmunized hα-syn tg mice were run in a minigel, multichannel apparatus (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. Each channel on the membrane was separated and incubated individually with diluted serum from each of the vaccinated mice. Serum dilution was adjusted to 1:1000 for all samples based on serum titers. Purified recombinant hα-syn was used as a standard, and all channels were normalized to this as a control. Channels were then incubated with secondary rabbit anti-mouse antibody (Vector Laboratories, Burlingame, CA) followed by I^{125}-tagged protein A (ICN Pharmaceuticals, Costa Mesa, CA) (Alford et al., 1994). Blots were imaged and analyzed with the PhosphorImager (Molecular Dynamics, Piscataway, NJ). For the second, to determine the specificity of the antibodies, brain homogenates from nontg animals, CFA-treated hα-syn tg mice, and immunized hα-syn tg mice were analyzed by WB with serum from CFA-treated mice and high relative affinity serum from hα-syn vaccinated tg mice. Blots were imaged and analyzed on a VersaDoc XL imaging apparatus (BioRad, Hercules, CA). For the third, serial vibratome sections from an untreated hα-syn tg mouse were incubated in diluted serum from each of the treated mice (adjusted to 1:1000) followed by biotinylated horse anti-mouse IgG (1:100, Vector), Avidin D-horseradish peroxidase (HRP, 1:200, ABC Elite, Vector), and reacted with diaminobenzidine tetrahydrochloride (DAB) containing 0.001% H_2O_2. After microscopic examination, sections were scored according to the cellular compartment labeled (neuronal cell bodies, synapses, and inclusions) and the degree of IR (0 = none; 1 = very mild, 2 = mild, 3 = moderate, 4 = intense).

Epitope Mapping of hα-Syn Antibodies

The epitopes recognized by hα-syn antibodies were determined by an ELISA that measures the binding of an antibody to overlapping linear peptides that covered the entire hα-syn sequence. C-terminally biotinylated peptides with sequences of hα-syn (Mimotopes, San Diego, CA) were prepared as 15 aa long peptides with an overlap of 12 residues and a step of 3 residues per peptide. To run the assay, these biotinylated peptides were coated down overnight at 5 nM onto ELISA plates precoated with streptavidin (Pierce, Rockford, IL), followed by washing and incubation for 1 hr with diluted serum samples. Serum samples with titers lower than 5000 were diluted 1:1000 for this incubation. After another washing step, the bound antibodies were detected using species-specific secondary antibodies conjugated to HRP in a colorimetric ELISA format.

Tissue Processing

Following NIH guidelines for the humane treatment of animals, mice were anesthetized with chloral hydrate and flush-perfused transcardially with 0.9% saline. Briefly, the right hemibrain was frozen and homogenized for determinations of hα-syn IR by WB (Masliah et al., 2000). The left hemibrain was fixed in 4% paraformaldehyde (PFA) and serially sectioned with the vibratome (Leica, Wetzlar, Germany) for ICC analysis.

Synaptosomal Preparation, Membrane and Cytosolic Fractionation, and Immunoblot Analysis

Synaptosomal fractions were prepared essentially as previously described (Dodd et al., 1981a; Dodd et al., 1981b). After resuspension in 0.32 M sucrose, synaptosomal fractions were analyzed by SDS-PAGE on a 10% tris-acetate polyacrylamide gel (NuPAGE, In-

vitrogen) and transferred onto Immobilon membranes (Millipore). For further analysis, total brain homogenates were separated into membrane and cytosolic fractions, prepared essentially as previously described (Hashimoto et al., 2002). The cytosolic and membrane fractions were analyzed by SDS-PAGE on 4%–12% Bis-Tris gels and transferred onto Immobilon membranes (Millipore).

Immunoblots with synaptosomal, cytosolic, and membrane fractions were probed with an affinity-purified rabbit polyclonal antibody against hα-syn (72-10, 1:5000) (Masliah et al., 2000) or with primary antibodies against synaptophysin (1:500, Chemicon, Temecula, CA) or actin (1:1000, Chemicon), followed by secondary goat anti-rabbit or anti-mouse IgG tagged with HRP (1:5000, Santa-Cruz Biotechnology, Inc., Santa Cruz, CA). Blots were visualized by ECL and analyzed with a VersaDoc XL imaging apparatus (BioRad).

Neuropathological and Immunocytochemical Analysis

Briefly, as previously described (Masliah et al., 2000), serially sectioned, free-floating, blind-coded vibratome sections were incubated overnight at 4°C with an anti-hα-syn-specific antibody (72-10, 1:500). To analyze the effects of the immunization in glial cell activation, sections were labeled with a mouse monoclonal antibody against the astroglial marker GFAP (1:500, Chemicon) or a rabbit polyclonal antibody against the microglial marker Iba-1 (1:1000, Wako Chemicals, Richmond, VA). Incubation with the primary antibodies was followed by biotinylated goat anti-rabbit IgG or horse anti-mouse IgG (1:100, Vector), Avidin D-HRP (1:200, ABC Elite, Vector), and reaction with DAB tetrahydrochloride containing 0.001% H_2O_2. Sections were analyzed with the Quantimet 570C (Leica) in order to determine the number of hα-syn-immunoreactive inclusions, astroglia, or microglia in the temporal cortex, a brain region often affected in patients with LBD (Hansen et al., 1990). For each case, three sections were analyzed, and the results were averaged and expressed as numbers per square millimeter.

Double-immunocytochemical analysis was performed as previously described (Hashimoto et al., 2004; Hashimoto et al., 2001) to determine the effects of vaccination on nerve terminal density and hα-syn accumulation in synapses. Vibratome sections were double-labeled with rabbit polyclonal antibodies against hα-syn (72-10, 1:5000) detected with Tyramide Red (1:2000, Roche, Switzerland) and with the mouse monoclonal antibody against synaptophysin (1:15, Chemicon), detected with a horse anti-mouse IgG FITC-tagged secondary antibody (1:75, Vector). For each case, sections were immunolabeled in duplicate and analyzed by LSCM and NIH Image 1.43 software to calculate the percent area of the neuropil covered by synaptophysin-immunoreactive terminals in the temporal cortex (Mucke et al., 2000) and the proportion of synaptophysin-immunoreactive terminals that were hα-syn positive (Hashimoto et al., 2004).

Control experiments were performed where sections were immunolabeled with an antibody against mα-syn (Masliah et al., 2000), detected with Tyramide Red (1:2000, Roche), or with a rabbit polyclonal antibody against β-syn (1:1000, Chemicon), detected with FITC-tagged goat anti-rabbit secondary antibody (1:75, Vector). In order to confirm the specificity of the primary antibodies, control experiments were performed where sections were incubated overnight in the absence of primary antibody (deleted), with the primary antibody preadsorbed for 48 hr with 20-fold excess of the corresponding peptide or with preimmune serum. To identify if antibodies generated by the vaccinated mice detected hα-syn in the tg animals, sections were double labeled with a horse anti-mouse FITC-tagged IgG (1:75, Vector) and a rabbit polyclonal antibody against hα-syn (72-10, 1:5000), detected with Tyramide Red (1:2000, Roche). To determine if hα-syn colocalized to lysosomes, sections were double labeled with an antibody against hα-syn (72-10, 1:5000), detected with Tyramide Red (1:2000, Roche), and a rabbit polyclonal antibody against cathepsin D (1:500, Calbiochem, San Diego, CA), detected with goat anti-rabbit FITC-tagged secondary antibody (1:75, Vector). All sections were processed simultaneously under the same conditions, and experiments were performed twice in order to assess the reproducibility of results. Sections were imaged with a Zeiss 63X (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss, Germany) with an attached MRC1024 LSCM system (BioRad, Wattford, UK) (Masliah et al., 2000). Images were analyzed with the image program NIH Image 1.43 to determine levels of mα-syn, β-syn, or cathepsin D IR.

Determination of In Vivo Antibody Recognition of Intracellular hα-Syn

To further validate whether antibodies against hα-syn recognized intracellular hα-syn, we generated an FITC-tagged antibody for injection into the brains of nontg and hα-syn tg (Line D) mice. For this purpose, a monoclonal antibody that recognizes aa 118–126 of hα-syn was generated in mice using recombinant hα-syn (clone 9E4, Elan Pharmaceuticals). This antibody was concentrated with a 10 kDa cutoff concentrator centrifuge tube (Millipore, Billerica, MA) and linked to the FITC molecule utilizing a FluoroTag FITC conjugation kit (Sigma) according to the manufacturer's instructions. The FITC-tagged hα-syn antibody or a control nonimmune FITC-tagged IgG was injected into the brains of these nontg (n = 5) and hα-syn tg (n = 5) mice (5-month-old). Briefly, as previously described (Marr et al., 2003), mice were placed under anesthesia on a Koft stereotaxic apparatus, and each mouse was injected with 3 μl of FITC-tagged anti-hα-syn antibody (approximately 0.5 μg/μl) or anti-mouse FITC-tagged IgG into the temporal cortex (using a 5 μl Hamilton syringe, 0.25 μl/min). After 3 days, mice were anesthetized with chloral hydrate and flush-perfused transcardially with 0.9% saline. Brains were fixed in 4% PFA for 48 hr and vibratomed. Sections from the brains of these mice were then analyzed by direct fluorescence or were coimmunolabeled with antibodies against hα-syn (72-10, 1:1000) or cathepsin D (1:500, Calbiochem), detected with secondary anti-rabbit Texas Red-tagged IgG (1:75, Vector). All sections were imaged with the LSCM as described before.

Statistical Analysis

After all results were obtained, the code was broken and statistical comparisons between groups were performed utilizing the two-tailed unpaired Student's t test. Linear regression analysis was performed to ascertain the relationship among variables. The Bonferroni correction was applied to account for multiple comparisons.

Supplemental Data

The authors' conflict of interest statement and Supplemental Data can be found online at http://www.neuron.org/cgi/content/full/46/6/857/DC1/.

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Neurobiology of Disease

Passive Amyloid Immunotherapy Clears Amyloid and Transiently Activates Microglia in a Transgenic Mouse Model of Amyloid Deposition

Donna M. Wilcock,[1,2] Amyn Rojiani,[1,3,4] Arnon Rosenthal,[5] Gil Levkowitz,[5] Sangeetha Subbarao,[5] Jennifer Alamed,[1,2] David Wilson,[1,2] Nedda Wilson,[1,2] Melissa J. Freeman,[1,2] Marcia N. Gordon,[1,2] and Dave Morgan[1,2]

[1]Alzheimer's Research Laboratory and Departments of [2]Pharmacology, [3]Interdisciplinary Oncology, and [4]Pathology, University of South Florida, Tampa, Florida 33612, and [5]Rinat Neuroscience Corporation, Palo Alto, California 94304

The role of microglia in the removal of amyloid deposits after systemically administered anti-Aβ antibodies remains unclear. In the current study, we injected Tg2576 APP transgenic mice weekly with an anti-Aβ antibody for 1, 2, or 3 months such that all mice were 22 months at the end of the study. In mice immunized for 3 months, we found an improvement in alternation performance in the Y maze. Histologically, we were able to detect mouse IgG bound to congophilic amyloid deposits in those mice treated with the anti-Aβ antibody but not in those treated with a control antibody. We found that Fcγ receptor expression on microglia was increased after 1 month of treatment, whereas CD45 was increased after 2 months of treatment. Associated with these microglial changes was a reduction in both diffuse and compact amyloid deposits after 2 months of treatment. Interestingly, the microglia markers were reduced to control levels after 3 months of treatment, whereas amyloid levels remained reduced. Serum Aβ levels and anti-Aβ antibody levels were elevated to similar levels at all three survival times in mice given anti-Aβ injections rather than control antibody injections. These data show that the antibody is able to enter the brain and bind to the amyloid deposits, likely opsonizing the Aβ and resulting in Fcγ receptor-mediated phagocytosis. Together with our earlier work, our data argue that all proposed mechanisms of anti-Aβ antibody-mediated amyloid removal can be simultaneously active.

Key words: Alzheimer's disease; antibody; behavior; microglia; immunization; amyloid β

Introduction

Reduction of brain amyloid after anti-Aβ immunotherapy was first demonstrated by Schenk and colleagues (1999). Their report showed that vaccination with $A\beta_{1-42}$ in the PDAPP transgenic mouse model of Alzheimer's disease dramatically reduced levels of Aβ deposits in the brain. Later it was shown that using the same vaccination protocol in APP + PS1 doubly transgenic mice (Morgan et al., 2000) and in TgCRND8 transgenic mice (Janus et al., 2000) not only reduced Aβ levels in the brain but also protected these mice from memory deficits. More recent studies have demonstrated that passive immunization consisting of direct anti-Aβ antibody injections not only results in dramatic reduction of Aβ levels (Bard et al., 2000; DeMattos et al., 2001) in the brain but also reverses memory deficits in transgenic mouse models of Alzheimer's disease (Dodart et al., 2002; Kotilinek et al., 2002).

The mechanisms by which immunotherapy acts remain unclear. Suggested mechanisms include microglial-mediated phagocytosis (Schenk et al., 1999; Wilcock et al., 2001, 2003, 2004), disaggregation of amyloid deposits (Solomon et al., 1997; Wilcock et al., 2003, 2004), and removal of Aβ from the brain by binding of circulating Aβ in plasma with the anti-Aβ antibodies, resulting in a concentration gradient from brain to plasma. This latter mechanism is also known as the peripheral sink hypothesis (DeMattos et al., 2001; Dodart et al., 2002; Das et al., 2003; Lemere et al., 2003).

We have previously reported that after intracranial anti-Aβ antibody injections into APP transgenic mice, there is a rapid removal of diffuse amyloid deposits apparently independent of microglial activation and also a later removal of compact amyloid deposits, which appears to require microglial activation (Wilcock et al., 2003). In fact, in a later study using the same model, administration of dexamethasone, which suppresses microglial activation, anti-Aβ antibody administration inhibited the removal of compact, thioflavine-S-positive amyloid deposits (Wilcock et al., 2004).

In this report, we show that weekly systemic administration of anti-Aβ antibodies for 1, 2, or 3 months results in a dramatic reduction of both diffuse and compact amyloid deposits. Associated with this reduction is a behavioral improvement using the Y maze task. After 1 month of treatment, there is a large induction of Fcγ receptor expression on microglia, and after 2 months of administration, there is an increase in CD45 expression indica-

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Correspondence should be addressed to Dr. Dave Morgan, Department of Pharmacology, University of South Florida, 12901 Bruce B. Downs Boulevard, MDC Box 9, Tampa, FL 33612. E-mail: dmorgan@hsc.usf.edu.

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tive of microglial activation. We have detected antibody binding to congophilic plaque in APP transgenic mice treated with an anti-Aβ antibody. We also observed a dramatic increase in circulating Aβ levels after 1 month of administration. Two months after administration, we observed a dramatic reduction in compact and diffuse deposits. After 3 months of administration, the microglia markers are down to control levels, whereas the compact and diffuse amyloid deposits remain reduced. These results demonstrate systemically administered anti-Aβ antibodies are accessing the brain, binding to amyloid deposits, and activating microglia. The data also show an increase in circulating Aβ in plasma, consistent with the peripheral sink hypothesis.

Materials and Methods

Experiment design. Singly transgenic APP Tg2576 mice were obtained from our breeding program at University of South Florida, started in 1996 (Holcomb et al., 1998). Twenty-two APP transgenic mice aged 19 months were assigned to one of four experimental groups. The first three groups received weekly intraperitoneal anti-Aβ antibody injections (antibody 2286, mouse monoclonal anti-human $Aβ_{28-40}$ IgG1; Rinat Neuroscience Corporation) for 1 month ($n = 6$), 2 months ($n = 9$), or 3 months ($n = 4$). The fourth group received weekly intraperitoneal anti-AMN antibody injections (2906; mouse monoclonal anti-*Drosophila* amnesiac protein IgG1; Rinat Neuroscience Corporation) for 3 months ($n = 3$). Twelve nontransgenic mice were assigned to one of two experimental groups. The first group received intraperitoneal anti-Aβ antibody injections for 3 months ($n = 4$). The second group received no treatment ($n = 3$). All mice were given a dose of 10 mg/kg of the appropriate antibody. Treatment of 1 and 2 month groups was delayed to insure the mice were killed at the same age (22 months). One week before killing and 1 d after the 5th, 9th, or 13th injection, mice were tested behaviorally using the Y maze task.

Behavioral testing. The Y maze is a three-arm maze with equal angles between all arms. Mice were initially placed within one arm, and the sequence and number of arm entries were recorded for each mouse over an 8 min period. The percentage of triads in which all three arms were represented (ABC, CAB, or BCA but not BAB) was recorded as an alternation to estimate short-term memory of the last arms entered. The total number of possible alternations is the number of arm entries minus two. Additionally, the number of arm entries serves as an indicator of activity.

Antibody purification. Antibody 2286 (mouse monoclonal anti-human $Aβ_{28-40}$ IgG1) and antibody 2906 (mouse monoclonal anti-*Drosophila* amnesiac protein IgG1) were purified from mouse ascites on AKTA instrumentation using protein A beads (MabSelect; Amersham Biosciences, Arlington Heights, IL). Briefly, ascites was filtered in a pyrogen-free 0.22 μm filter system (Corning, Corning, NY) and applied to a 20 ml bed volume in an XK16/20 column (Amersham Biosciences) after equilibrating the beads with 5 vol of binding buffer (0.6 M NaCl and 0.3 M glycine, pH 8.0). The column was washed with 3 vol of binding buffer, and the antibody was eluted in 4 vol of elution buffer (0.1 M Na citrate, pH 3.0) and held at low pH for 30 min for viral inactivation. The resulting eluant was neutralized with 0.1 vol of 1.0 M Tris, pH 9.5. The antibody was dialyzed into sterile PBS, pH 7.4, and the concentration was determined by reading absorbance at 280 nm. All buffers were made in pyrogen-free water.

Tissue preparation. On the day of killing, mice were weighed and overdosed with 100 mg/kg pentobarbital (Nembutal sodium solution; Abbott Laboratories, North Chicago, IL). Blood was collected and allowed to coagulate at 4°C for at least 1 hr before being centrifuged, and the serum was removed and stored at −80°C until required. The mice were then intracardially perfused with 25 ml of 0.9% sodium chloride. Brains were rapidly removed, and the right half of the brain was dissected and frozen for biochemistry, whereas the left half of the brain was immersion-fixed for 24 hr in freshly prepared 4% paraformaldehyde in 100 mM PO_4, pH 7.2, for histopathology. The latter hemibrains were then incubated for 24 hr in 10, 20, and 30% sucrose sequentially to cryoprotect them. Horizontal sections of 25 μm thickness were collected using a sliding microtome and stored at 4°C in Dulbecco's PBS with sodium azide, pH 7.2, to prevent microbial growth.

ELISA methods: Aβ and anti-Aβ antibody. For the Aβ assay, serum was diluted and incubated in 96-well microtiter plates (MaxiSorp; Nunc, Rosklide, Denmark), which were precoated with antibody 6E10 (Signet, Dedham, MA) at 5 μg/ml in PBS buffer, pH 7.4. The secondary antibody was biotinylated 4G8 (Signet) at a 1:5000 dilution. Detection was done using a streptavidin-horseradish peroxidase conjugate (Amersham Biosciences), followed by TMB substrate (KPL, Gaithersburg, MD). Standard curves of $Aβ_{1-40}$ (Global Peptide, Ft. Collins, CO) scaling from 6–400 pm were used.

The anti-Aβ antibody was dissociated from endogenous Aβ in serum as described previously (Li et al., 2004). Briefly, serum was diluted in dissociation buffer (0.2 M glycine HCl and 1.5% BSA, pH 2.5) and incubated at room temperature for 20 min. The sera were pipetted into the sample reservoir of a Microcon centrifugal device (10,000 molecular weight cutoff; YM-10; Millipore, Bedford, MA) and centrifuged at 8000 × *g* for 20 min. at room temperature. The sample reservoir was then separated from the flow-through, placed inverted into a second tube, and centrifuged at 1000 × *g* for 3 min. The collected solution containing the antibody dissociated from the Aβ peptide was neutralized to pH 7.0 with 1 M Tris buffer, pH 9.5. The dissociated sera were assayed by ELISA for antibody titer. $Aβ_{1-40}$ (Global Peptide)-coated 96-well microtiter plates (MaxiSorp; Nunc) were incubated with dissociated serum samples. A biotinylated goat-anti mouse IgG (heavy and light chain; Vector Laboratories, Burlingame, CA) at a 1:5000 dilution followed by peroxidase-conjugated streptavidin (Amersham Biosciences) was used to detect serum anti-Aβ binding activity.

Immunohistochemical methods. A series of eight equally spaced tissue sections 2.4 mm apart were randomly selected, spanning the entire brain, and stained using free-floating immunohistochemistry methods for total Aβ (rabbit polyclonal anti-pan-Aβ, 1:10,000; Bisource, Camarillo, CA), CD45 (rat anti-mouse CD45, 1:3000; Serotec, Raleigh, NC), and Fcγ receptors II and III (rat anti-mouse CD16 and CD32, 1:3000; BD PharMingen, San Diego, CA) as previously described (Gordon et al., 2002). Briefly, tissue was incubated in primary antibody overnight at room temperature. Sections were then washed and incubated in the appropriate biotinylated secondary antibody (for Aβ, goat anti-rabbit, 1:3000; for CD45 and FcγR, goat anti-rat, 1:1000; all Vector Laboratories) for 2 hr. After multiple washes, tissue was incubated in ABC (Vector Laboratories) for 1 hr. Color development was performed using 3,3′-diaminobenzidine (DAB; Sigma , St. Louis, MO) enhanced with nickelous ammonium sulfate (J. T. Baker Chemical Company, Phillipsburg, NJ) for CD45 and FcγR or without enhancement for Aβ. For immunostaining, some sections were omitted from the primary antibody to assess nonspecific immunohistochemical reactions.

Additional sections were also stained for mouse IgG using immunohistochemical methods similar to that described above. Briefly, sections were incubated overnight in a 1:3000 concentration of anti-mouse IgG conjugated to horseradish peroxidase (Sigma). The sections were then washed and incubated for 5 min in 100 ml of Tris-buffered saline containing 50 mg of DAB (Sigma), 500 mg of nickelous ammonium sulfate (J. T. Baker Chemical Company), and 100 μl of 30% hydrogen peroxide to produce a purple-black color reaction product. The sections were then mounted on slides and counterstained with a 0.2% Congo red solution in 80% ethanol to assess the localization of positive mouse IgG stain with compact amyloid deposits.

A set of sections was also mounted and stained using 0.2% Congo red solution in NaCl-saturated 80% ethanol. Another set of sections was also mounted and stained using 4% thioflavine-S (Sigma) for 10 min.

The immunohistochemical reaction product on all sections was measured using Image-Pro Plus version 4.5 software (Media Cybernetics, Silver Spring, MD). One region of the frontal cortex for all sections from each animal was analyzed, and an average of six or seven sections was taken to give a value for each animal. Three regions of the hippocampus were analyzed on approximately four or five sections where hippocampus was present: cornu ammonis 1 (CA1), CA3, and dentate gyrus. These regions were analyzed both individually to yield an average per region and also combined to give an overall value for the hippocampus for each

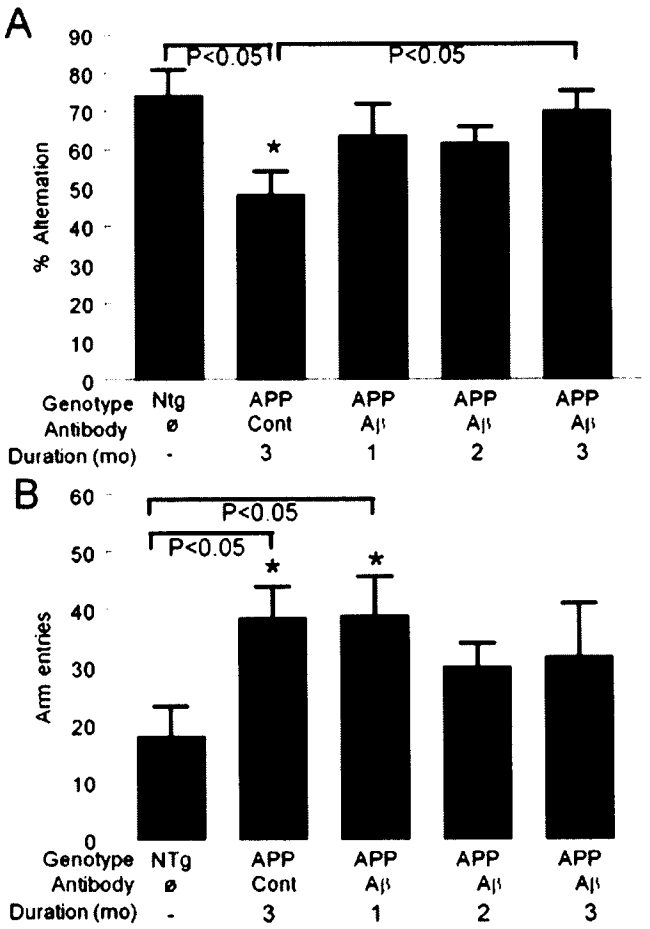

Figure 1. Y maze behavioral improvement after systemic anti-Aβ antibody administration. *A*, Percent alternation for nontransgenic (NTg) mice receiving no treatment (ø), APP transgenic mice (APP) receiving the control antibody (Cont) for 3 months, and APP transgenic mice (APP) receiving the anti-Aβ (Aβ) antibody for 1, 2, or 3 months. $*p < 0.05$ when compared with nontransgenic untreated mice and APP transgenic mice receiving the anti-Aβ antibody for 3 months. *B*, Number of arm entries for nontransgenic mice receiving no treatment, APP transgenic mice receiving the control antibody for 3 months, and APP transgenic mice receiving the anti-Aβ antibody for 1, 2, or 3 months. $*p < 0.05$ when compared with nontransgenic untreated mice.

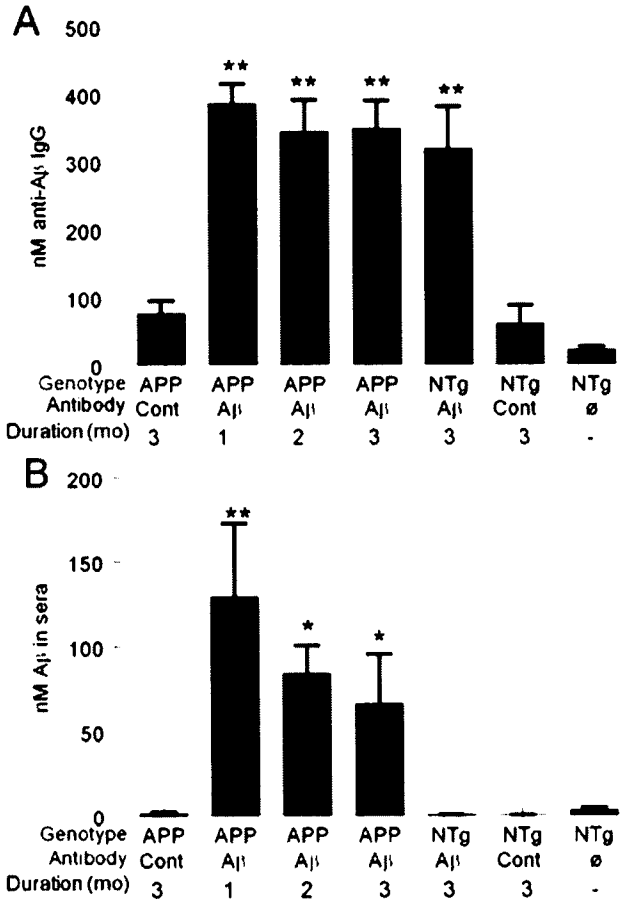

Figure 2. Increased serum levels of anti-Aβ antibody and Aβ after anti-Aβ antibody administration. *A*, Amounts of circulating anti-Aβ antibodies in APP transgenic mice (APP) receiving either the control antibody (Cont) for 3 months or the anti-Aβ antibody (Aβ) for 1, 2, or 3 months, nontransgenic (NTg) mice receiving either the control antibody or the anti-Aβ antibody for 3 months, and nontransgenic mice receiving no treatment. $**p < 0.001$ compared with APP mice given control antibody injections. *B*, Amounts of circulating Aβ in sera in APP transgenic mice receiving either the control antibody for 3 months or the anti-Aβ antibody for 1, 2, or 3 months, nontransgenic mice receiving the either control antibody (Cont) or the anti-Aβ antibody for 3 months, and nontransgenic mice receiving no treatment. $**p < 0.01$; $*p < 0.05$.

animal. This ensured that there was no regional bias in the hippocampal values. These same analysis methods were also used to evaluate the Congo red stain. To assess possible treatment-related differences, the values for each treatment group were analyzed by one-way ANOVA followed by Fisher's least significant difference (LSD) means comparisons. Nontransgenic mice showed no treatment-related differences in any histological analyses, so these groups were pooled.

Data analysis. Percent alternation and arm entry numbers from the Y maze behavior task were analyzed using one-way ANOVA followed by Fisher's LSD means comparisons using StatView software version 5.0.1 (SAS Institute Inc., Cary, NC). Nontransgenic mice showed no treatment-related differences in any behavioral analyses, so these groups were pooled. ELISA values for serum Aβ levels and circulating antibody levels were analyzed using one- ANOVA followed by Fisher's LSD means.

Results

Transgenic APP mice given control antibody injections showed significantly reduced Y maze alternation when compared with the nontransgenic mice (Fig. 1*A*). This reduced alternation was reversed in the APP transgenic mice receiving weekly anti-Aβ antibody injections for 3 months. This group of mice was indistinguishable from the nontransgenic animals and showed significantly increased alternation compared with the APP transgenic mice receiving the control antibody (Fig. 1*A*). The APP transgenic mice given weekly anti-Aβ antibody injections for either 1 or 2 months were intermediate between nontransgenic and transgenic mice given control antibodies and not significantly different from either group. Nontransgenic mice also made significantly fewer arm entries than the APP transgenic mice receiving control antibody injections, indicating hyperactivity in the APP transgenic mice. The APP transgenic mice receiving anti-Aβ antibody injections for 2 and 3 months did not exhibit this hyperactivity and were not significantly different from any other treatment groups (Fig. 1*B*).

One day after anti-Aβ antibody administration, anti-Aβ antibodies were detected in serum at high levels (400 nM) after 1 month of administration. This level of antibody in the serum was the same after 2 or 3 months of administration, with no apparent accumulation of the antibody (Fig. 2*A*). Associated with high anti-Aβ antibody levels in serum at 1 month was a dramatic increase in circulating Aβ levels in serum. APP transgenic mice receiving the control antibody had only 1.5 nM circulating Aβ in

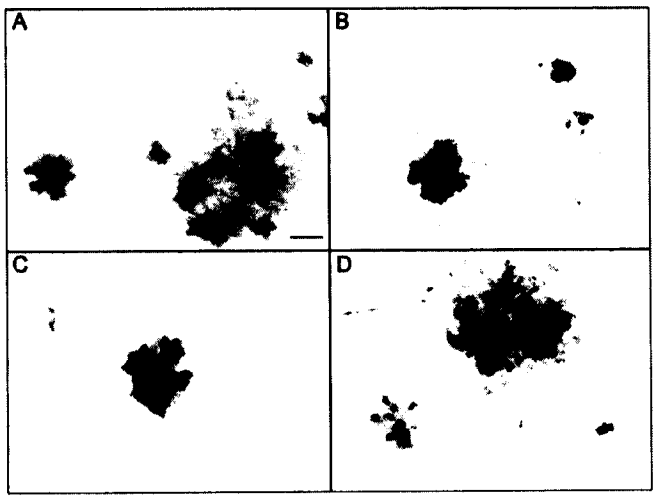

Figure 3. Mouse IgG immunohistochemistry showing antibody binding to Congophilic plaques in anti-Aβ antibody-treated mice but not control antibody-treated mice. *A–D*, Anti-mouse IgG-HRP immunohistochemistry counterstained with Congo red to detect compact amyloid deposits. *A*, Representative amyloid deposit and associated anti-mouse IgG immunostaining (black) in the hippocampus of a mouse injected with the control antibody for 3 months. *B–D*, Representative amyloid deposit (red) associated with anti-mouse IgG immunostaining (black) in the hippocampus of a mouse injected with the anti-Aβ antibody for 1 month (*B*), 2 months (*C*), or 3 months (*D*). Magnification, 200×. Scale bar, 25 μm.

plasma compared with APP transgenic mice receiving the Aβ antibody for 1 month, which had 130 nM circulating Aβ in plasma, an almost 100-fold increase (Fig. 2*B*). Despite similar levels of anti-Aβ antibody at 1, 2, or 3 months of administration, circulating Aβ levels declined between 1 and 2 months. They also showed a slight decline between 2 and 3 months of administration, although with both 2 and 3 months of administration, circulating Aβ levels were still significantly elevated compared with APP transgenic mice receiving the control antibody (Fig. 2*B*).

After systemic administration of anti-Aβ antibodies weekly for 1 month, staining for mouse IgG could be detected on plaques throughout the brains of APP transgenic mice (Fig. 3*B*). The staining was the most intense where plaque load was greatest: the hippocampus and frontal cortex. This staining was not observed in APP transgenic mice receiving the control antibody (Fig. 3*A*). It should be noted that staining with higher concentrations of anti-mouse IgG-HRP did show staining of plaques in both control-treated and anti-Aβ treated APP transgenic mice. Staining for mouse IgG was still present and slightly more intense around the plaques that remained after 2 (Fig. 3*C*) and 3 (Fig. 3*D*) months of treatment.

Total Aβ immunohistochemistry in the APP transgenic mice receiving control antibody (Fig. 4*A*) showed a few intensely stained deposits, suggesting compacted amyloid deposits along with more numerous diffuse deposits. There was a concentration of deposits around the hilus of the hippocampus as well as the molecular layers of Ammon's horn. This was a typical amount and distribution of Aβ for APP transgenic mice of this age, as previously described (Hsiao et al., 1996; Gordon et al., 2002). After 1 month of weekly anti-Aβ antibody injections, there appeared to be a slight reduction in Aβ immunohistochemistry in the hippocampus (Fig. 4*B*), although this was not statistically significant (Fig. 4*E*). The reduction appeared to be primarily diffuse deposits, with most of the compact amyloid deposits remaining (Fig. 4*B*). After 2 months of weekly anti-Aβ antibody injections, we observed a dramatic reduction in Aβ immunohistochemistry, which appeared to be both compact and diffuse amyloid deposits from the hilus and dentate gyrus regions of the hippocampus as well as the pyramidal cell regions, with only a few deposits remaining, often in the vicinity of the hippocampal fissure and outer molecular layers (Fig. 4*C*). This reduction in Aβ load at 2 months was ~60% in the hippocampus and ~55% in the frontal cortex (Fig. 4*E*) (hippocampus, $p < 0.001$; frontal cortex, $p < 0.005$). Total Aβ levels remained reduced after 3 months of treatment but did not appear to decrease any further (Fig. 4*D*,*E*).

Congo red staining detects only compact amyloid deposits in the β pleated sheet structure. There were far fewer Congo red-positive amyloid deposits than Aβ deposits detected by total Aβ immunohistochemistry. Congo red-positive deposits were located primarily along the fissure of the hippocampus as well as the CA1/subiculum region in APP transgenic mice receiving the control antibody (Fig. 5*A*). There was no reduction in Congophilic deposits 1 month after treatment in either the hippocampus (Fig. 5*B*,*E*) or the frontal cortex (Fig. 5*E*). After 2 months of treatment, there was a significant reduction in both the number and size of Congophilic deposits in both the hippocampus (Fig. 5*C*,*E*) and frontal cortex (Fig. 5*E*). This reduction was ~60% in the frontal cortex and ~50% in the hippocampus (Fig. 5*E*) (frontal cortex, $p < 0.005$; hippocampus, $p < 0.01$). There was a small further reduction between 2 and 3 months, which was ~30% in the hippocampus and frontal cortex (Fig. 5*E*). Thioflavine-S staining was also measured and confirmed the Congo red data, showing the same reductions in the stained area as did Congo red (data not shown).

Immunohistochemical staining for Fcγ receptors II and III in APP transgenic mice receiving control antibody treatment for 3 months showed only very faint staining of microglia in close association with amyloid deposits (Fig. 6*A*). After 1 month of anti-Aβ antibody administration, there was a dramatic induction of Fcγ receptors II and III on microglia. The microglia expressing the Fcγ receptors after 1 month of treatment were not only associated with amyloid deposits but also diffusely distributed (Fig. 6*B*). This induction averaged 100-fold in the hippocampus (Fig. 6*B*,*E*) ($p < 0.05$) and frontal cortex (Fig. 6*E*) ($p < 0.05$). Fcγ receptor expression levels fell only slightly between 1 and 2 months of treatment, although this expression was once again concentrated on microglia around remaining amyloid deposits (Fig. 6*D*). Induction remained ~100-fold in the hippocampus (Fig. 6*E*) ($p < 0.05$) and frontal cortex (Fig. 6*E*) ($p < 0.05$). After 3 months of treatment, Fcγ receptor expression was reduced to levels observed in APP transgenic mice receiving the control antibody (Fig. 6*E*), although it appeared to be increased in microglia around the few remaining amyloid deposits (Fig. 6*D*).

CD45, a protein-tyrosine phosphatase, is normally moderately expressed on microglia around amyloid deposits in aged APP transgenic mice and is a commonly used marker for microglial activation. This moderate expression was observed in the APP transgenic mice receiving control antibody treatment for 3 months (Fig. 7*A*,*E*). After 1 month of treatment, we observed an increase in CD45 expression on microglia surrounding amyloid deposits in both the hippocampus (Fig. 7*B*,*F*,*I*) and frontal cortex (Fig. 7*I*). Although expression in the hippocampus was ~2.5 times that observed in control-treated APP transgenic mice (Fig. 7*I*) (not significant) and twice the values found in the frontal cortex of control animals, the elevation was not statistically significant (Fig. 7*I*). After 2 months of anti-Aβ antibody treatment, there was an additional increase in CD45 expression on microglia not only surrounding the amyloid deposits but also diffusely dis-

tributed throughout the amyloid-containing brain regions (Fig. 7*C*,*G*). It is possible that this more widespread activation is in association with diffuse amyloid deposits, although we cannot confirm this. The increased expression was ~3.5 times that observed in control-treated mice in the hippocampus (Fig. 7*I*) ($p < 0.05$) and 3 times in the frontal cortex (Fig. 7*I*) ($p < 0.01$). After 3 months of anti-Aβ antibody treatment, CD45 expression remained at the same levels as that observed after 1 month of treatment (Fig. 7*I*); however, the microglia were still diffusely distributed, with fewer microglia around deposits compared with 1 or 2 months of treatment (Fig. 7*D*,*H*).

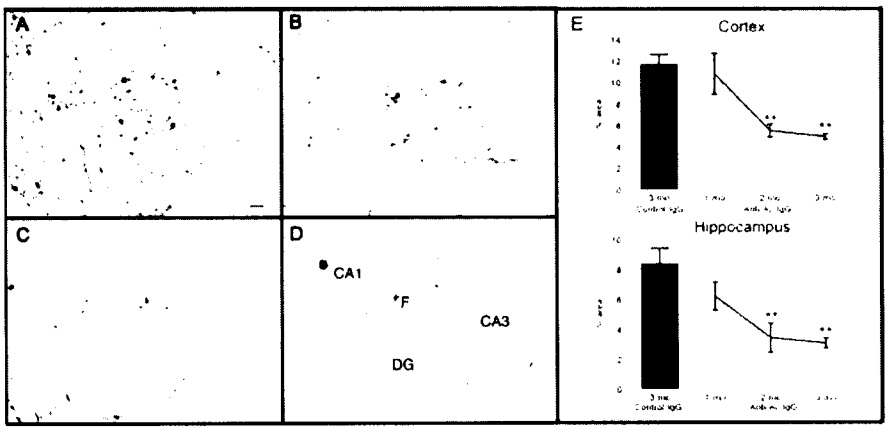

Figure 4. Total Aβ immunohistochemistry is reduced after 2 months of systemic anti-Aβ antibody administration. *A–D*, Total Aβ immunohistochemistry in the hippocampus of APP transgenic mice receiving the control antibody for 3 months (*A*; percent area for this section was 9.12%), the anti-Aβ antibody for 1 month (*B*; percent area for this section was 6.84%), the anti-Aβ antibody for 2 months (*C*; percent area for this section was 3.23%), or the anti-Aβ antibody for 3 months (*D*; percent area for this section was 2.49%). Magnification, 40×. Scale bar, 120 μm. *E*, Quantification of the percent area occupied by the Aβ-positive stain in the frontal cortex and hippocampus. The single bar shows the value for APP transgenic mice receiving the control antibody for 3 months. The line shows the values for APP transgenic mice receiving the anti-Aβ antibody for 1, 2, and 3 months. $^{**}p < 0.01$. CA1, Cornu ammonis 1; CA3, cornu ammonis 3; DG, dentate gyrus; F, hippocampal figure.

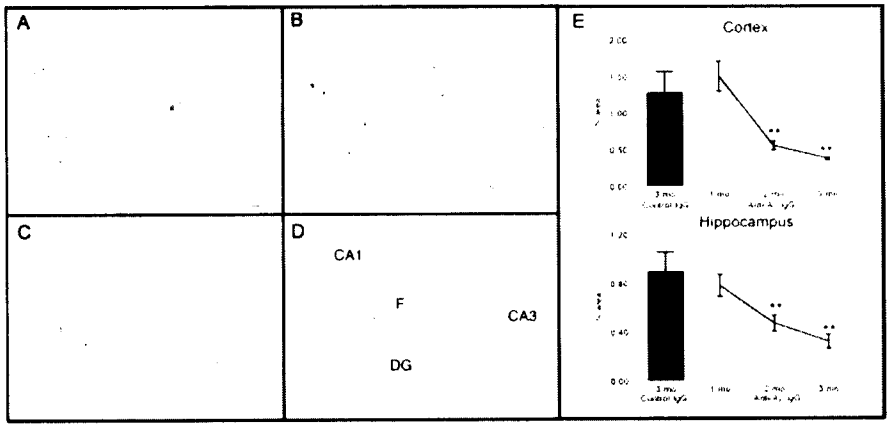

Figure 5. Congophilic compact amyloid plaques are reduced after 2 months of anti-Aβ antibody administration. *A–D*, Congo red staining in the hippocampus of APP transgenic mice receiving the control antibody for 3 months (*A*), the anti-Aβ antibody for 1 month (*B*), the anti-Aβ antibody for 2 months (*C*), or the anti-Aβ antibody for 3 months (*D*). Magnification, 40×. Scale bar, 120 μm. *E*, Quantification of the percent area occupied by the Congo red-positive stain in the frontal cortex and hippocampus. The single bar shows the value for APP transgenic mice receiving the control antibody for 3 months. The line shows the values for APP transgenic mice receiving the anti-Aβ antibody for 1, 2, and 3 months. $^{**}p < 0.01$. CA1, Cornu ammonis 1; CA3, cornu ammonis 3; DG, dentate gyrus; F, hippocampal figure.

Discussion

The data presented here suggest that peripherally administered anti-Aβ antibodies entered the brain, bound to congophilic amyloid plaques, and led to removal of deposited amyloid. In support of the argument that anti-Aβ antibodies entered the brain, we found mouse IgG marking the remaining congophilic amyloid plaques of APP transgenic mice administered the anti-Aβ antibody but no IgG in APP transgenic mice administered the control antibody. This difference was best discerned when low titers of the anti-mouse IgG-HRP were used. Seabrook et al. (2004) also reported immunohistochemical labeling of amyloid deposits for mouse IgG after passive immunization but detected signals in both immunized and nonimmunized mice. It is unclear whether lower anti-mouse IgG concentrations might have revealed selective staining in anti-Aβ-treated animals. These data confirm in paraformaldehyde-fixed tissue the observations of Bard et al. (2000), who used unfixed cryostat sections.

Associated with the presence of the antibody in the brain after 1 month of treatment was a dramatic activation of Fcγ receptor expression on microglia, further arguing that anti-Aβ antibodies entered the brain and opsonized the amyloid deposits. Later, after 2 months of treatment, we observed an increase in CD45 expression on microglia, indicating activation of these cells beyond the level normally associated with amyloid deposits. It has previously been shown that after active immunization with $A\beta_{1-42}$ in humans, anti-Aβ antibodies are present in CSF, in some instances equal to the serum concentration, suggesting some penetration into the brain from the periphery (Hock et al., 2002). It has also been shown that 0.1% of an intravenous injection of a radiolabeled anti-Aβ antibody crosses the blood–brain barrier of SAMP8 mice (Banks et al., 2002). Thus, accumulating data indicate that circulating antibodies can access the brain parenchyma, which has important implications not only for the use of immunotherapy in Alzheimer's disease but also for other diseases in which immunotherapy is being pursued, such as Creutzfeldt–Jakob disease (Manuelidis, 1998; Sigurdsson et al., 2003) and neural infections associated with human immunodeficiency virus (McMichael and Hanke, 2003).

Associated with the changes in microglial markers was a significant reduction in both compact and diffuse amyloid deposits after 2 months of treatment; these remained reduced after 3 months of treatment. Removal of Aβ deposits from the brain appeared to be a gradual process. We did not observe significant reductions in either diffuse or compact amyloid deposits after 1 month of weekly anti-Aβ antibody treatment. After 2 months of treatment, there was a dramatic reduction in total Aβ immunohistochemistry, Congo red staining, and thioflavine-S staining, suggesting removal of both diffuse and compact amyloid deposits. There appeared to be no accumulation of the injected antibody because serum anti-Aβ antibody levels were the same re-

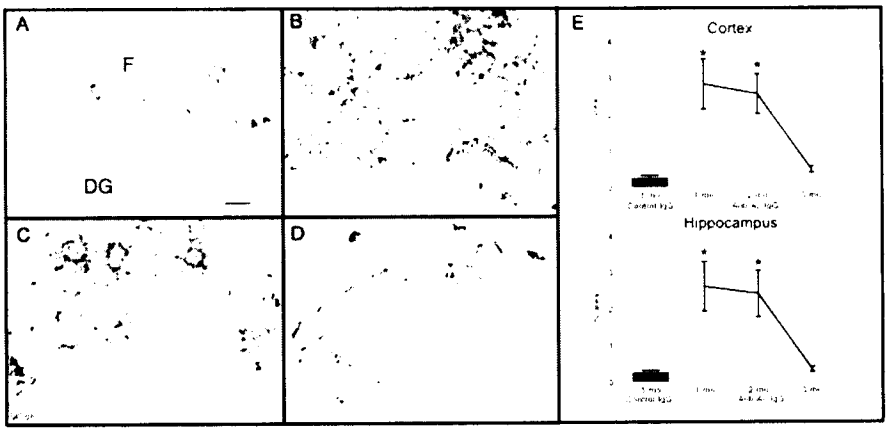

Figure 6. Fcγ receptor expression on microglia is increased after 1 month of anti-Aβ antibody treatment and remains increased after 2 months of treatment. *A–D*, Fcγ receptor immunohistochemistry in the hippocampus of APP transgenic mice receiving the control antibody for 3 months (*A*), the anti-Aβ antibody for 1 month (*B*), the anti-Aβ antibody for 2 months (*C*), or the anti-Aβ antibody for 3 months (D). Magnification, 100×. Scale bar, 50 μm. *E*, Quantification of the percent area occupied by the Fcγ receptor-positive stain in the frontal cortex and hippocampus. The single bar shows the value for APP transgenic mice receiving the control antibody for 3 months. The line shows the values for APP transgenic mice receiving the anti-Aβ antibody for 1, 2, and 3 months. $^*p < 0.05$. DG, Dentate gyrus; F, hippocampal fissure.

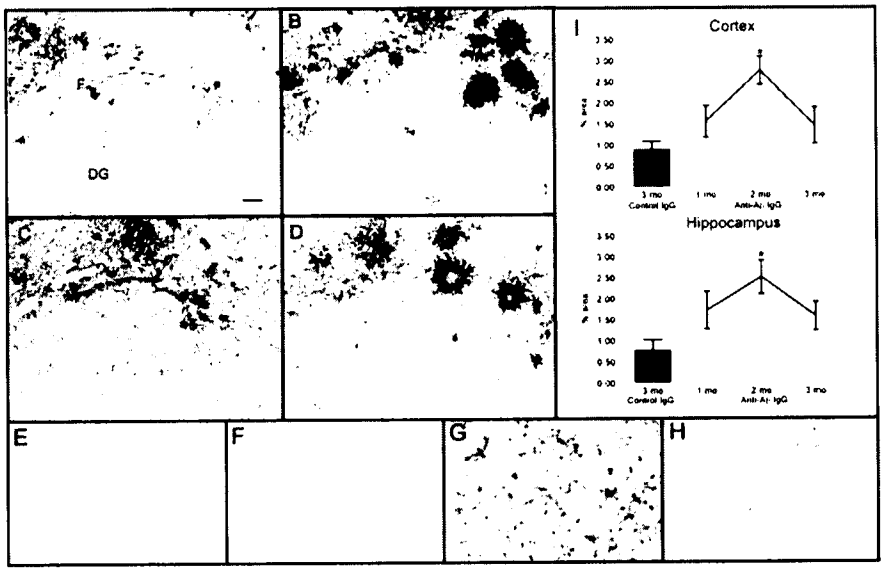

Figure 7. CD45 expression on microglia is increased after 2 months of anti-Aβ antibody treatment. *A–D*, CD45 immunohistochemistry in the hippocampus of APP transgenic mice receiving the control antibody for 3 months (*A*), the anti-Aβ antibody for 1 month (*B*), the anti-Aβ antibody for 2 months (*C*), or the anti-Aβ antibody for 3 months (*D*). F, Hippocampal fissure; DG, dentate gyrus. Magnification, 100×. Scale bar, 50 μm. *E–H*, Magnified images of non-amyloid-containing areas from *A–D*, showing CD45 immunohistochemistry in the hippocampus of APP transgenic mice receiving the control antibody for 3 months (*E*), the anti-Aβ antibody for 1 month (*F*), the anti-Aβ antibody for 2 months (*G*), or the anti-Aβ antibody for 3 months (*H*). *I*, Quantification of the percent area occupied by the CD45-positive stain in the frontal cortex and hippocampus. The single bar shows the value for APP transgenic mice receiving the control antibody for 3 months. The line shows the values for APP transgenic mice receiving the anti-Aβ antibody for 1, 2, and 3 months. $^*p < 0.05$.

gardless of the duration of treatment. This would suggest that this time-dependent removal of amyloid deposits was not occurring because of increasing antibody levels; rather, it appears that some mobilization of removal mechanisms must be present for some time before significant removal is apparent.

An early feature we observed was the increase in Fcγ receptor II and III (CD16 and CD32) expression on microglia, which was apparent after 1 and 2 months of treatment. The murine Fcγ receptors II and III share a high affinity for IgG1 antibodies (the isotype used in the current study) as well as IgG2a (Gessner et al., 1998). Following this increased Fcγ receptor expression was an increase in CD45 expression on microglia after 2 months of treatment. CD45 is a protein-tyrosine phosphatase, which is elevated with microglial activation. In this study, it appears that the increase in CD45 expression represents a further activation step from that seen after 1 month of treatment, at which we observed the increased Fcγ receptor expression. After 3 months of treatment, both Fcγ receptor and CD45 expression on microglia were reduced to control levels, possibly because of the substantial reduction in amyloid deposits. It is important to note that if we had looked at only the 3 month point, we would not have detected the activation of the microglia by CD45 or, likely, by other markers such as Mac-1 (Das et al., 2003).

We have previously observed a similar loss of microglia activation after intracranial antibody administration (Wilcock et al., 2003) and active immunization (Wilcock et al., 2001). Three days after a single injection of anti-Aβ antibody in the frontal cortex and hippocampus, we observed an increase in CD45 expression; however, 7 d after injection, the CD45 expression was reduced to control levels, in parallel with clearance of the Aβ deposits (Wilcock et al., 2003). This suggests that the reduced microglial activation could possibly be attributable to the clearance of most amyloid plaques. It is also conceivable that the microglia could be undergoing apoptosis attributable to the robust activation, as has been described previously by Liu et al. (2001), when microglia are overactivated by lipopolysaccharide. An alternative explanation could be tolerance of the microglia to antibody-opsonized Aβ. We have previously shown a reduction in microglial reaction in an active immunization model using doubly transgenic APP + PS1 mice. After five monthly inoculations, we observed a significant increase in CD45 expression; however, after nine monthly inoculations, CD45 levels were comparable with those of control animals despite continued high antibody titer levels (Wilcock et al., 2001).

Because of the inflammatory adverse effects seen in a human clinical trial of the active immunization by Elan Pharmaceuticals (Ferrer et al., 2004), it could be suggested that the microglial activation observed in this study was attributable to an immune response unrelated to opsonization of Aβ by the antibody. To evaluate whether this was the case, we examined the thalamus and cerebellum, which do not contain any amyloid deposits, for any increase in CD45 or Fcγ receptor expression and did not observe any such increase. Thus, it appears that the microglial activation is specific to amyloid-containing brain regions and is likely a specific response to opso-

nized Aβ as opposed to a general nonspecific inflammatory reaction.

The data presented here extend our previous observations of the benefits of active anti-Aβ immunization on learning and memory (Morgan et al., 2000). We show that passive immunization with anti-Aβ antibodies for 3 months reduced amyloid deposits and improved behavioral performance, as indicated by a significant increase in alternation in the Y maze as well as a decrease in the number of arm entries. The arm entry data suggest that there is not a complete reversal of the increased activity. There is a trend toward some improvement in alternation at the 1 month point (although not significant) despite no reduction in total Aβ immunohistochemistry. Such improvements may reflect rapid reductions of an Aβ pool (oligomeric?) closely linked to memory impairments yet not easily detected by immunohistochemistry. This phenomenon was previously described by Dodart et al. (2002) and Kotilinek et al. (2002), who showed rapid reversal of memory deficits in transgenic mice after passive immunization without significant reduction in brain Aβ.

The results described above indicating entry of the anti-Aβ antibody into the brain and activation of microglia suggest that some opsonization of Aβ is likely stimulating microglial involvement in the clearance of Aβ deposits. This is consistent with the phagocytosis mechanisms of amyloid removal put forward by the Elan Phrmaceuticals group (Schenk et al., 1999; Bard et al., 2000, 2003). Our earlier work with direct injection of the anti-Aβ antibody into the brain suggests two mechanisms: one not requiring an Fc component or activation of microglia, which can clear diffuse Aβ, and a second that requires the Fc domain and activation of microglia (Wilcock et al., 2003, 2004). It is conceivable that the first non-Fc-requiring mechanism is analogous to the catalytic dissolution mechanism described by Solomon et al. (1997). The diffuse material, whatever its state of oligomerization, may be more accessible to this action of anti-Aβ antibodies. Finally, at all durations of antibody exposure, we observed a dramatic increase in circulating Aβ levels in plasma. This is consistent with a role for the peripheral sink mechanism (DeMattos et al., 2001; Dodart et al., 2002; Lemere et al., 2003) in the reduction of CNS Aβ after passive immunization.

We conclude that our studies using antibody 2286, in aggregate, provide support for all three major proposed mechanisms of anti-Aβ antibody action in lowering brain amyloid. It is essential to recognize that these mechanisms are not mutually exclusive and are likely to be synergistic if multiple mechanisms are elicited by a single antibody or serum. It is also important to recognize that not all monoclonal antibodies need work via all three mechanisms. Both isotype and epitope selectivity could regulate which anti-Aβ action is dominant for a specific antibody. These studies also do not speak to other immune system-related actions that might underlie the benefits (or adverse effects) of active immunization. Nonetheless, given the preliminary data that anti-Aβ immunotherapy may stabilize cognitive function in Alzheimer's patients (Hock et al., 2003) and the consistent reversal of the phenotype found in APP transgenic mice by such approaches, these results support further development of optimal strategies for using anti-Aβ immunotherapy as a treatment for Alzheimer's dementia.

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Intracranially Administered Anti-Aβ Antibodies Reduce β-Amyloid Deposition by Mechanisms Both Independent of and Associated with Microglial Activation

Donna M. Wilcock,[1] Giovanni DiCarlo,[1] Debbi Henderson,[1] Jennifer Jackson,[1] Keisha Clarke,[1] Kenneth E. Ugen,[2] Marcia N. Gordon,[1] and Dave Morgan[1]

Departments of [1]Pharmacology and [2]Medical Microbiology and Immunology, Alzheimer's Research Laboratory, University of South Florida, Tampa, Florida 33612

Active immunization against the β-amyloid peptide (Aβ) with vaccines or passive immunization with systemic monoclonal anti-Aβ antibodies reduces amyloid deposition and improves cognition in APP transgenic mice. In this report, intracranial administration of anti-Aβ antibodies into frontal cortex and hippocampus of Tg2576 transgenic APP mice is described. The antibody injection resulted initially in a broad distribution of staining for the antibody, which diminished over 7 d. Although no loss of immunostaining for deposited Aβ was apparent at 4 hr, a dramatic reduction in the Aβ load was discernible at 24 hr and was maintained at 3 and 7 d. A reduction in the thioflavine-S-positive compact plaque load was delayed until 3 d, at which time microglial activation also became apparent. At 1 week after the injection, microglial activation returned to control levels, whereas Aβ and thioflavine-S staining remained reduced. The results from this study suggest a two-phase mechanism of anti-Aβ antibody action. The first phase occurs between 4 and 24 hr, clears primarily diffuse Aβ deposits, and is not associated with observable microglial activation. The second phase occurs between 1 and 3 d, is responsible for clearance of compact amyloid deposits, and is associated with microglial activation. The results are discussed in the context of other studies identifying coincident microglial activation and amyloid removal in APP transgenic animals.

Key words: Alzheimer's disease; Aβ; antibody; immunization; intracranial; phagocytosis

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive deficits. There are several pathological characteristics to the disease process, including congophilic amyloid plaques that contain the β-amyloid peptide (Aβ) and intracellular inclusions of neurofibrillary tangles that consist of hyperphosphorylated tau protein. Another characteristic of AD is the initiation and proliferation of a brain-specific inflammatory response that consists of activated microglia and astrocytes. Amyloid deposition is thought to be the key step in the pathogenesis of AD (Selkoe, 1991; Hardy and Selkoe, 2002); this is why development of potential therapies focuses on clearance of amyloid.

Vaccination with $A\beta_{1-42}$ was first described by Schenk et al. (1999). Their report demonstrated that active immunization with $A\beta_{1-42}$ in the PDAPP transgenic mouse reduced levels of Aβ deposits dramatically. This immunization protected APP+PS1 transgenic mice (Morgan et al., 2000) and TgCRND8 transgenic mice (Janus et al., 2000) from memory deficits. More recent studies showed that treatment with a passive immunization regimen that consisted of anti-Aβ antibodies resulted in a dramatic reduction in Aβ (Bard et al., 2000; DeMattos et al., 2001) and reversal of memory deficits (Dodart et al., 2002; Kotilinek et al., 2002) in the PDAPP mouse.

In this experiment, we show that intracranially administered anti-Aβ antibodies have both an early microglia-independent and a later, possibly microglia-dependent mechanism of action. Aβ levels were dramatically reduced 24 hr after administration in the absence of microglial activation. However, 72 hr after antibody administration, thioflavine-S-positive compact plaques were reduced concomitant with a striking activation of microglia.

Materials and Methods

Transgenic Tg2576 APP mice (Hsiao et al., 1996) were obtained after breeding of Tg2576 APP mice with line 5.1 PS1 mice (Duff et al., 1996), which yielded four different genotypes: nontransgenic, transgenic APP, transgenic PS1, and doubly transgenic APP + PS1 mice. Animals were provided food and water *ad libitum* and were kept on a 12 hr light/dark cycle; they were housed in groups if possible until before the surgery, when they were all singly housed until they were killed. We used two cohorts of mice in this study, the first cohort of 19-month-old APP mice ($n = 16$) and the second cohort of 16-month-old APP mice ($n = 22$).

Mice from the first cohort all received anti-Aβ antibodies (Biosource, Camarillo, CA; mouse anti-Aβ IgG_1, recognizing amino acids 1–16). Mice from the second group were assigned to groups that received anti-Aβ antibodies, control antibody (anti-HIV, 1D6; K. Ugen, Department of Medical Microbiology and Immunology, University of South Florida, Tampa, FL) ($n = 5$), or vehicle (0.02% thimerosal in PBS; Sigma-Aldrich, St. Louis, MO) ($n = 5$). All mice were injected in both the frontal cortex and hippocampus of the right hemisphere, whereas the left hemisphere remained untreated as an internal control. Those mice that received anti-Aβ antibodies were assigned survival times of 4 ($n = 5$), 24 ($n = 7$), 72 ($n = 8$), or 168 ($n = 6$) hr. Mice receiving either control

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Correspondence should be addressed to Dave Morgan, 12901 Bruce B. Downs Boulevard, MDC 9, Tampa, FL 33612. E-mail: dmorgan@hsc.usf.edu.

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antibody or vehicle were examined after a 72 hr survival time. A third group of untreated 17-month-old APP mice ($n = 5$) were killed without having been injected or manipulated to assess differences between the right and left sides of the brain.

On the day of surgery, the mice were weighed, anesthetized with isoflurane, and placed in a stereotaxic apparatus (51603 dual manipulator laboratory standard; Stoelting, Wood Dale, IL). A midsagittal incision was made to expose the cranium, and two burr holes were drilled with a dental drill over the right frontal cortex and hippocampus to the following coordinates: cortex, anteroposterior, + 1.5 mm; lateral, −2.0 mm; hippocampus, anteroposterior, −2.7 mm; lateral, −2.5 mm, all taken from bregma. A 26 gauge needle attached to a 10 μl syringe (Hamilton, Reno, NV) was lowered 3 mm ventral to bregma, and a 2 μl injection was made over a 2 min period. The incision was cleaned with saline and closed with surgical staples.

On the day they were killed, the mice were overdosed with pentobarbital 100 mg/kg (Nembutal sodium solution; Abbott Laboratories, North Chicago, IL) and perfused intracardially with 25 ml of 0.9% sodium chloride and 50 ml of freshly prepared 4% paraformaldehyde, pH 7.4. The brains were collected and postfixed for 24 hr in 4% paraformaldehyde. The brains were then incubated for 24 hr in 10, 20, and 30% sucrose sequentially to cryoprotect them. Horizontal sections of 25 μm thickness were then collected with a sliding microtome and stored at 4°C in Dulbecco's PBS buffer with sodium azide to prevent microbial growth. Six to eight sections 100 μm apart were selected that spanned the injection site and were stained by free-floating immunohistochemistry methods for total Aβ (rabbit antiserum primarily reacting with the N terminus of the Aβ peptide; 1:10,000), CD45 (Serotec, Raleigh, NC; 1:3000), and major histocompatibility complex class II (MHC-II; BD PharMingen, Palo Alto, CA; 1:3000) as described previously (Gordon et al., 2002). For immunostaining, some sections were omitted from the primary antibody to assess nonspecific immunohistochemical reactions. Immunohistochemical methods were used to stain for the injected antibody with anti-mouse IgG conjugated to horseradish peroxidase (Sigma-Aldrich; 1:1000). Adjacent sections were mounted on slides and stained with 4% thioflavine-S (Sigma-Aldrich) for 10 min. Selected sections stained for CD45 were counterstained for Congo red (Sigma-Aldrich) to detect amyloid deposits on these sections.

The immunohistochemical reaction product on all stained sections was measured with a videometric V150 image analysis system (Oncor, San Diego, CA) in the injected area of cortex and hippocampus and corresponding regions on the contralateral side of the brain. Data are presented as the average ratio of injected side to noninjected side for Aβ, thioflavine-S, and CD45, whereas data for MHC-II are expressed as area occupied by positive stain, because many values on the contralateral side were close to zero.

To assess possible treatment-related differences, the measurement for either cortex or hippocampus of each subject were analyzed by ANOVA with StatView software version 5.0.1 (SAS Institute Inc., Raleigh, NC) followed by Fisher's least significant difference means comparisons.

Results

Immunohistochemistry against mouse IgG was performed to trace the diffusion of anti-Aβ antibodies after injection into the hilus of the dentate gyrus. The injected anti-Aβ antibody showed diffuse distribution throughout the entire hippocampus at 4 hr, with a focal concentration in the outer molecular areas of the dentate and Ammons' horn near the hippocampal fissure (Fig. 1*A*). By 24 hr, the diffuse pattern remained broad, but the focal concentration began shifting toward the granule cell layers of the dentate gyrus (Fig. 1*B*). At 72 hr, staining for the injected antibody was lighter and became concentrated at the granule cell layer of the dentate gyrus (Fig. 1*C*). Interestingly, by the 1 week time point, the injected antibody staining had cleared for the most part, with some residual staining in the outer molecular layer of the ventral (ventricular) blade of the dentate gyrus and the glial limitans. A similar time course of staining was seen in the frontal cortex (data not shown).

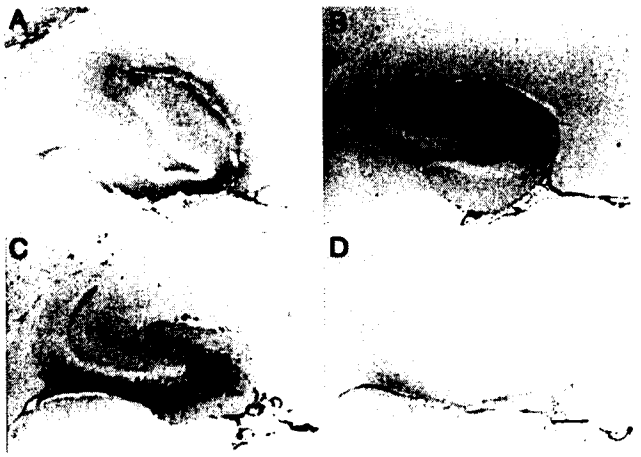

Figure 1. Time course of injected anti-Aβ antibody distribution in the hippocampus from 4 hr to 7 d. Immunohistochemical staining is shown for the injected antibody in the hippocampus at 4 (*A*), 24 (*B*), 72 (*C*), and 168 (*D*) hr. Orientation and locations of hippocampal subregions are as in Figure 2*D*. Magnification, 40×. Scale bar, 120 μm. A high-resolution color version of this micrograph can be obtained by e-mail from D. Morgan (dmorgan@hsc.usf.edu).

Aβ immunohistochemistry in APP transgenic mice resembled that reported previously by others and ourselves (Hsiao et al., 1996; Gordon et al., 2002). In both cortex (Fig. 2*A*) and hippocampus (Fig. 2*C*), there were a few intensely stained deposits and a number of smaller, less intensely stained deposits. In previous work, we found the intensely stained Aβ deposits were usually also stained with thioflavine-S or Congo red (Holcomb et al., 1998; Gordon et al., 2001), which indicates they were analogous to compact deposits containing fibrillar amyloid, whereas the less intense deposits were analogous to diffuse, nonfibrillar deposits observed commonly in AD tissue. Whereas the deposits were distributed fairly uniformly within the cortex, in the hippocampus they were concentrated in the outer molecular layers of the dentate gyrus and Ammon's horn (Fig. 2*C*). The subiculum also appeared more rich in deposits than other areas.

The injection of anti-Aβ antibody into brain did not result in a rapid loss of signal in postmortem immunohistochemical reactions, because we did not observe a change in Aβ staining 4 hr after injection in either cortex (Fig. 3*A*) or hippocampus (Fig. 3*B*). However, Aβ staining was reduced at the injection sites in frontal cortex and hippocampus 24 hr after administration of anti-Aβ antibody (Fig. 2*B*,*D*, respectively) and remained reduced to roughly the same extent through the 1 week time point (Fig. 3). The reduction in the frontal cortex was 60% compared with both the 4 hr time points and the two control groups of vehicle and anti-HIV antibody (Fig. 3*A*; $p < 0.001$). The reduction in the hippocampus was 50% compared with the 4 hr time points and the control groups (Fig. 3*B*; $p < 0.005$).

An interesting phenomenon was that the ratio of Aβ staining on the right to left sides in untreated mice was >1, which indicates more Aβ deposition on the right side than the left (Fig. 3). It appears that this pattern of Aβ deposition is a consistent property of the APP mice. The Aβ distribution seen in the mice administered control injections at 3 d and anti-Aβ antibody at the 4 hr time point is the typical distribution found in APP transgenic mice of this age.

As expected, considerably fewer deposits were stained with thioflavine-S than by Aβ immunohistochemistry. Nonetheless, the regional distribution of these deposits approximately paral-

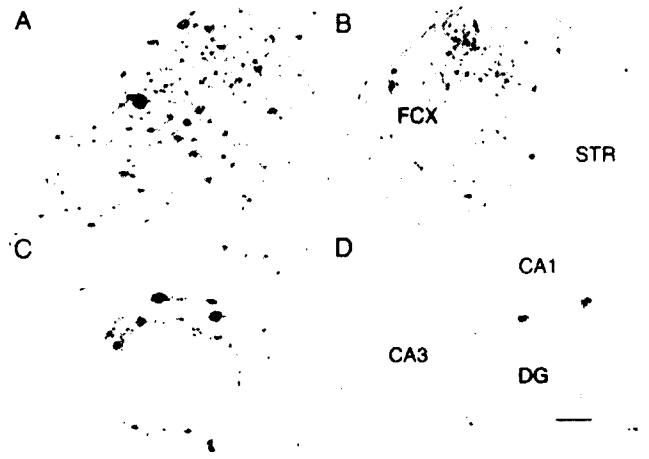

Figure 2. Reduction in Aβ immunohistochemistry 1 d after anti-Aβ antibody injections. Immunohistochemical staining is shown for Aβ in the frontal cortex (*A*, *B*) and hippocampus (*C*, *D*). *A* and *C* are from an animal injected with control antibody, whereas animals for which stains are shown in *B* and *D* received the anti-Aβ antibody. Magnification, 40×. Scale bar, 120 μm. *B*, FCX, frontal cortex; STR, striatum. *D*, CA1, cornu ammonis 1; CA3, cornu ammonis 3; DG, dentate gyrus. A high-resolution color version of this micrograph can be obtained by e-mail from D. Morgan (dmorgan@hsc.usf.edu).

leled that of Aβ-positive deposits in the cortex and hippocampus (Fig. 4*A*,*C*). In contrast to the Aβ, thioflavine-S-positive staining at the injection site was not reduced until 72 hr after administration of anti-Aβ antibody (Fig. 4*B*,*D*) and remained reduced at the 1 week time point (Fig. 5). The reduction in frontal cortex was 80% compared with the 4 and 24 hr time points and the control groups (Fig. 5*A*; $p < 0.001$). The reduction in hippocampus was 60% compared with both the 4 and 24 hr time points and the control groups (Fig. 5*B*; $p < 0.005$).

In untreated mice, activated microglia stained with CD45 or MHC-II antibodies were found only in the immediate periphery of compacted plaques. In the injected control groups, some microglial activation was detected at the 72 hr survival time by CD45 antibodies, and this was restricted primarily to the injection site (Fig. 6*A*,*C*, arrows; quantified in Fig. 7). Very little staining for CD45 was detected on the uninjected side of the brain, which led to inflated right-to-left ratios with relatively small increases in staining. MHC-II had a lower overall level of expression than that of CD45 and was mostly unaffected in mice administered control injections (Fig. 8*A*,*C*; quantified in Fig. 9).

In contrast, 72 hr after the injection of anti-Aβ antibodies, the microglial activation detected with CD45 antibodies was more widespread, detected not only at the injection site but also away from the injection site in the frontal cortex (Fig. 6*B*) and throughout the dentate gyrus, with a concentration within the granule cell layer at the 72 hr time point (Fig. 6*D*). MHC-II staining revealed a similar pattern, although not as extensive as that found with CD45 staining (Fig. 8*B*,*C*).

Quantification of these results indicated that the injection of anti-Aβ antibodies increased expression of the microglial marker CD45 significantly only at the 72 hr time point compared with all other time points and control groups in both cortex (Fig. 7*A*; $p < 0.005$) and hippocampus (Fig. 7*B*; $p < 0.005$). Also, the injection of anti-Aβ antibodies increased the expression of the microglial marker MHC-II at the 72 hr time point compared with all other time points and control groups in both cortex (Fig. 9*A*; $p < 0.01$) and hippocampus (Fig. 9*B*; $p < 0.005$). The expression of CD45 and MHC-II in the frontal cortex at the anti-Aβ injection site increased more than eightfold over that of all other time points,

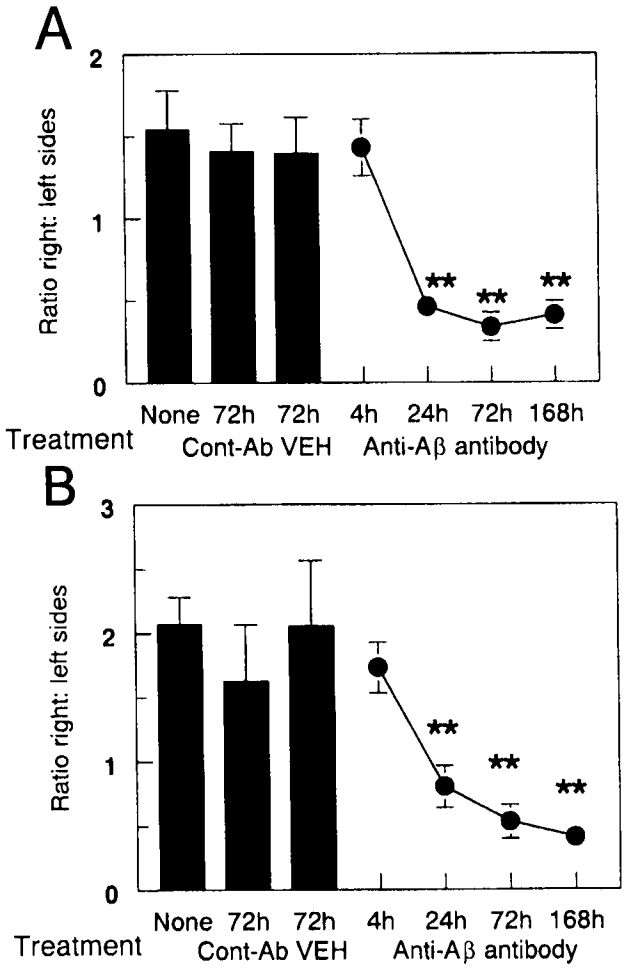

Figure 3. Quantification of reduced Aβ load after anti-Aβ antibody injections. Data are expressed as the ratio of Aβ staining in the injected hemisphere to the control hemisphere. The three bars on the left indicate the Aβ load in the untreated group (None) and the vehicle (VEH) and anti-HIV antibody (Cont-Ab) groups at 72 hr. The line shows the ratio of Aβ immunohistochemical staining at 4, 24, 72, and 168 hr survival times. Reduced Aβ load was observed in the frontal cortex (*A*) and hippocampus (*B*) at 24, 72, and 168 hr compared with 4 hr and both control groups (**$p < 0.005$).

including 1 week, and both of the control groups. The expression of CD45 in the hippocampus at the anti-Aβ injection site increased more than twofold, whereas the increase in expression of MHC-II was more than eightfold. As in our previous work, there was considerable variability among samples with both microglial markers; however, all anti-Aβ-injected animals had values that were greater than the means for the control groups.

There were few remaining amyloid deposits near the injection sites in the anti-Aβ antibody-injected mice at 72 hr (Figs. 4, 5). These residual deposits were relatively faint when stained with Congo red and were contacted by rounded, CD45-positive microglial cells (Fig. 10*B*). In contrast, the more abundant amyloid deposits on the contralateral side and in the control animals were contacted by microglia with long processes that were stained for CD45, whereas the cell body stained only faintly for this marker of microglial activation (Fig. 10*A*).

Discussion

Intracranial anti-Aβ antibody injections reduced Aβ load substantially in the vicinity of the injection in both anterior cortex and hippocampus over a 7 d time frame. By 4 hr after the injec-

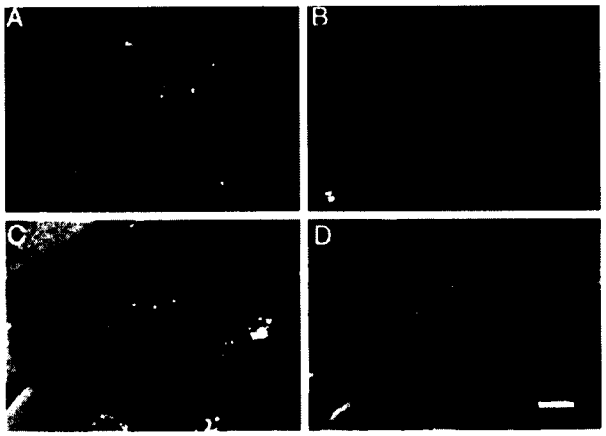

Figure 4. Reduction in thioflavine-S staining 3 d after anti-Aβ antibody injections. Thioflavine-S staining is shown in frontal cortex (*A*, *B*) and hippocampus (*C*, *D*). Mice in *A* and *C* received control antibody, whereas those in *B* and *D* received anti-Aβ antibody. Magnification, 40×. Scale bar, 120 μm. Orientation and locations of major subregions are as in Figure 2, *B* and *D*. A high-resolution color version of this micrograph can be obtained by e-mail from D. Morgan (dmorgan@hsc.usf.edu).

tion, there was a broad distribution of injected antibody that filled a volume of ~0.5 mm^3, as estimated from anti-IgG immunohistochemistry. In addition to the broad pattern of diffusion, the antibody was concentrated in the outer molecular layers of Ammon's horn and the dentate gyrus, a zone that mostly overlaps with the distribution of Aβ staining in transgenic mice of this age (Fig. 2*C*). Thus, it appears the injected antibody was binding to *in situ* Aβ at this early time point but was also spread throughout the hippocampus. By 24 hr, there was a reduction in the Aβ immunostaining in the vicinity of the antibody injection in both cortex and hippocampus. This reduction in Aβ load is unlikely to be an artifact caused by the injected antibody masking the epitope of the primary antibody used for immunohistochemistry, because the reduced load was not detected 4 hr after administration, and by 24 hr, the injected IgG appeared to be concentrated closer to the granule cell region than the outer molecular layer in the hippocampus. Furthermore, the stoichiometry of injected antibody (13 pmol) to Aβ in deposits (estimated at 250 pmol in 0.5 mg; Chapman et al., 1999) is likely too low to interfere substantially with the histochemical reaction. This early reduction in Aβ load occurred in the absence of the expression of microglial activation markers CD45 and MHC-II. Although this does not preclude some rapid response of the microglia, it does suggest that the role of microglia is qualitatively different at this early postsurvival time from when markers of activation are being expressed.

Between 24 and 72 hr after injection of anti-Aβ antibodies, there were parallel reductions in fibrillar amyloid deposits detected by thioflavine-S and increases in microglial activation evaluated by CD45 and MHC-II staining. Although control injections of anti-HIV antibody and vehicle caused some elevation of the CD45 marker, activation was restricted to the immediate vicinity of the injection site and was likely caused by mechanical injury associated with needle insertion and fluid compression of the tissue. Occasionally, in the anti-Aβ antibody-injected mice, some remaining wisps of amyloid could be found in the vicinity of the antibody injection at 72 hr, and these were in contact with rounded CD45-immunopositive cells suggestive of phagocytic microglia or macrophages. Also at the 72 hr time point, there was a concentration of staining for both the injected antibody and the microglia near the granule cell layer of the dentate gyrus. The temporal association of fibrillar amyloid loss with microglial activation suggests some causal role for microglial activation in this process. One possibility is that between 1 and 3 d, activated microglia near the deposits in the outer molecular layer phagocytosed opsonized amyloid via Fc receptor- or complement-mediated mechanisms and migrated toward the granule cell layer. CD45-positive microglia could be detected readily in the outer molecular layer near the fissure at 3 d, although they were concentrated most heavily near the granule cell layer at that time point (Fig. 6*D*). A second option is that after dissolution of the Aβ deposits, the antibodies diffused to the granule cell region independently of the microglia. Possibly, the fibrillar deposits simply required more time to dissolve than the more diffuse material. More detailed time course studies of the period between 1 and 3 d coupled with immunoelectron microscopy will likely be required to decide between these options. Remarkably, the microglial activation was terminated rapidly and returned to normal levels by the 1 week time point in conjunction with a significant reduction in staining for the injected IgG and Aβ.

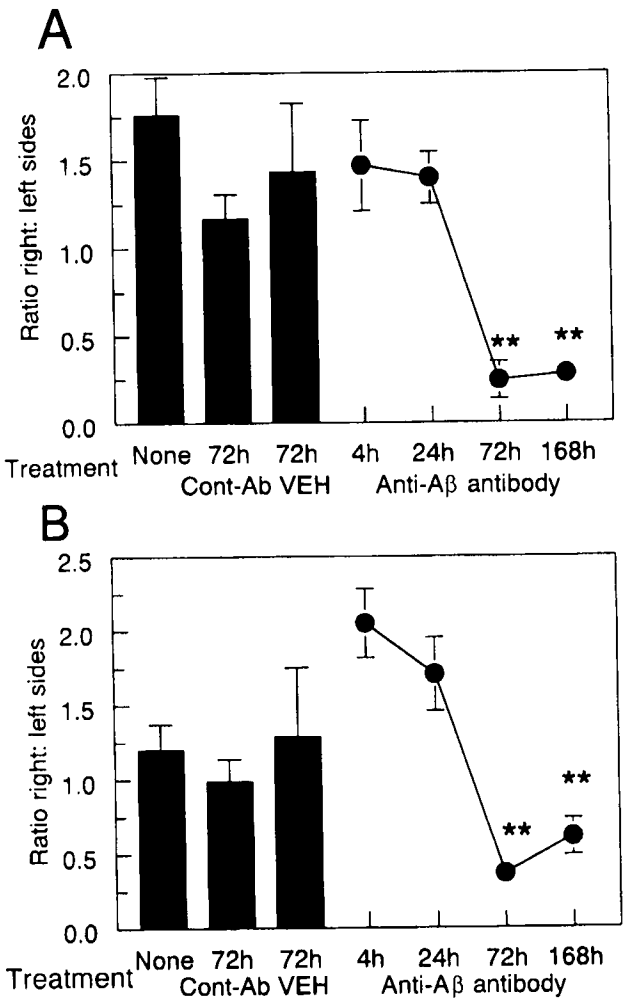

Figure 5. Anti-Aβ antibody injections result in a reduction of thioflavine-S-positive plaques. Data are expressed as the ratio of thioflavine-S staining in the injected hemisphere to the control hemisphere. The three bars show thioflavine-S-positive staining in the untreated group (None) and the vehicle (VEH) and anti-HIV antibody (Cont-Ab) groups at 72 hr. The line shows the ratio of thioflavine-S staining at 4, 24, 72, and 168 hr survival times. Reduced thioflavine-S staining was observed in the frontal cortex (*A*) and hippocampus (*B*) at 72 and 168 hr compared with 4 and 24 hr and both control groups (**$p < 0.005$).

An accumulating body of evidence finds an association between microglial activation and amyloid reductions in transgenic

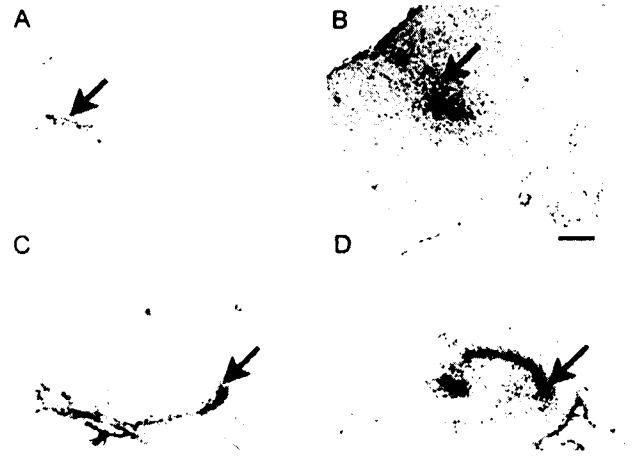

Figure 6. CD45 immunohistochemistry is increased 3 d after anti-Aβ antibody injections. CD45 immunohistochemistry is shown in frontal cortex (*A*, *B*) and hippocampus (*C*, *D*). Mice in *A* and *C* received control antibody, whereas those in *B* and *D* received anti-Aβ antibody. Magnification, 40×. Scale bar, 120 μm. Arrows indicate the site of injection identified from the needle tract. A high-resolution color version of this micrograph can be obtained by e-mail from D. Morgan (dmorgan@hsc.usf.edu).

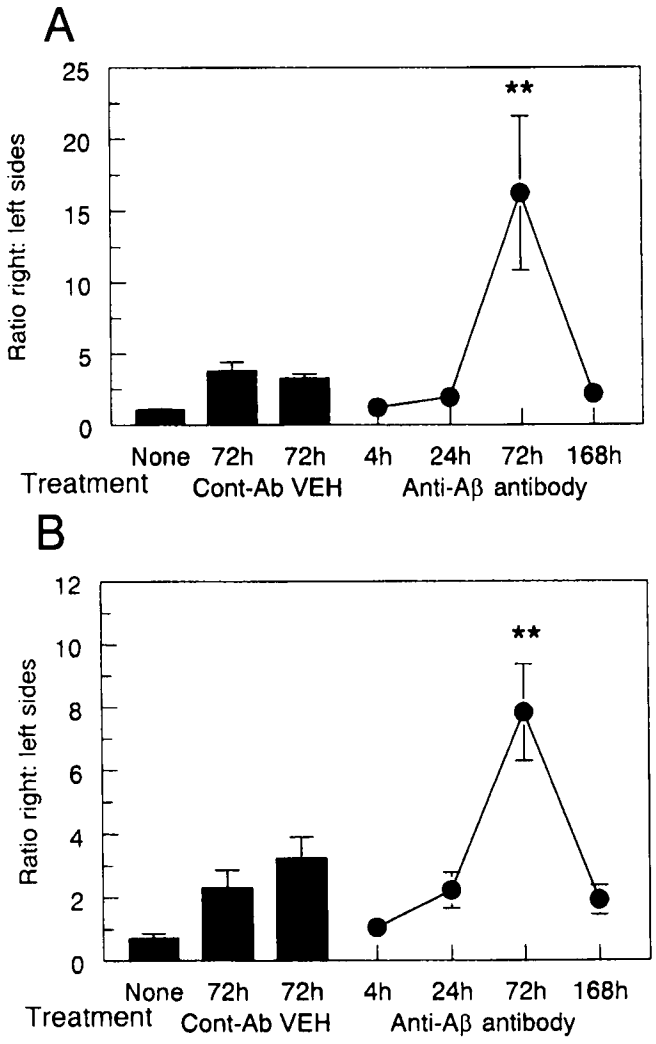

Figure 7. Anti-Aβ antibody injections result in increased CD45 immunohistochemistry 3 d after injection. Data are expressed as the ratio of CD45 staining in the injected hemisphere to the control hemisphere. The three bars indicate CD45 expression in the untreated group (None) and the vehicle (VEH) and anti-HIV antibody (Cont-Ab) groups at 72 hr. The line shows the ratio of CD45 staining at 4, 24, 72, and 168 hr survival times. Increased CD45 staining was observed in the frontal cortex (*A*) and hippocampus (*B*) at 72 hr compared with 4, 24, and 168 hr and both control groups (**$p < 0.005$).

mouse models of amyloid deposition. Schenk et al. (1999) noted in the first study evaluating Aβ vaccines that the clearance of amyloid was associated with enhanced microglial activity around the remaining deposits. Wilcock et al. (2001) mostly confirmed this observation in a different transgenic model. Nakagawa et al. (2000) unexpectedly found that fluid percussion injury activates microglia and results in reduced amyloid deposition as mice grow older. Lim et al. (2001) noted that transgenic mice treated with curcumin had a reduced amyloid load but an increase in the activation state of microglia surrounding plaques. Similarly, Jantzen et al. (2002) found a reduced amyloid load in transgenic mice treated with a nitric oxide-releasing nonsteroidal anti-inflammatory drug, NCX-2216, which was also associated with increased microglial activation. Wyss-Coray et al. (2001) found that the crossing of APP transgenic mice with mice that overexpressed TGF-β led to increased microglial activation and reduced amyloid loads. Conversely, Wyss-Coray et al. (2002) found that blocking of complement activation with soluble complement receptor-related protein Y overexpression diminished the microglial reaction in APP transgenic mice and led to elevated amyloid loads. DiCarlo et al. (2001) attempted to activate microglia directly by injecting lipopolysaccharide and found that this was associated with clearance of Aβ in the vicinity of the injection. However, Qiao et al., (2001) injected lipopolysaccharide chronically into young transgenic mice before normal amyloid deposition and found that it could stimulate Aβ deposition. It is also the case that careful serial section electron microscopy failed to detect internalized amyloid in microglia associated with amyloid deposits in untreated APP23 transgenic mice (Stalder et al., 2001), although mice treated to provoke microglial activation have yet to be examined. Nonetheless, there is a growing body of literature that associates the activation of microglia with a reduction in Aβ deposition in the transgenic mouse models.

A number of studies have demonstrated that cultured microglial cells are capable of internalizing Aβ1–42 aggregates (Paresce et al., 1996; Webster et al., 2001). Aβ can also be cleared from unfixed brain sections by anti-Aβ antibodies in a microglia-dependent manner (Bard et al., 2000). Direct imaging of amyloid deposits *in vivo* by multiphoton microscopy has shown clearance of plaque after application of an anti-Aβ antibody in association with an upregulation of activated microglia (Bacskai et al., 2001). Suggested alternative mechanisms to microglial phagocytosis include a physical interaction between antibody and Aβ that results in disaggregation of deposits, which was demonstrated *in vitro* with monoclonal anti-Aβ antibodies (Solomon, 2001). Consistent with this idea, Bacskai et al. (2002) demonstrated recently that $F(ab')_2$ fragments prepared from an anti-Aβ antibody reduced amyloid deposits as effectively as the intact antibody when applied topically to the cortex of transgenic mice through a craniotomy. Although there was no measurement of microglial activation in this study, it is plausible that this occurred in the absence of microglial involvement. This antibody-mediated dissolution hypothesis is consistent with the early phase of Aβ reduction described here and may still be found to be responsible for the second phase of fibrillar deposit reduction.

A major unresolved question is how this antibody-mediated clearance of Aβ might apply to the human condition. Increasingly, AD has been argued to involve inflammation as a component of its pathogenesis (McGeer and McGeer, 2001). The early

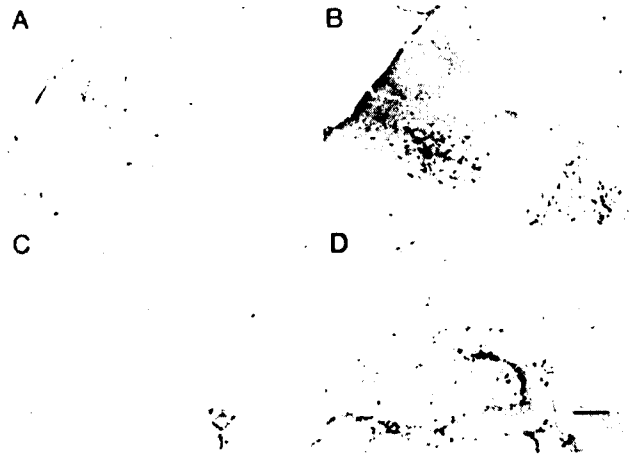

Figure 8. MHC-II immunohistochemistry is increased 3 d after anti-Aβ antibody injections. MHC-II immunohistochemistry is shown in frontal cortex (*A*, *B*) and hippocampus (*C*, *D*). Mice in *A* and *C* received control antibody, whereas those in *B* and *D* received anti-Aβ antibody. Magnification, 40×. Scale bar, 120 μm. A high-resolution color version of this micrograph can be obtained by e-mail from D. Morgan (dmorgan@hsc.usf.edu).

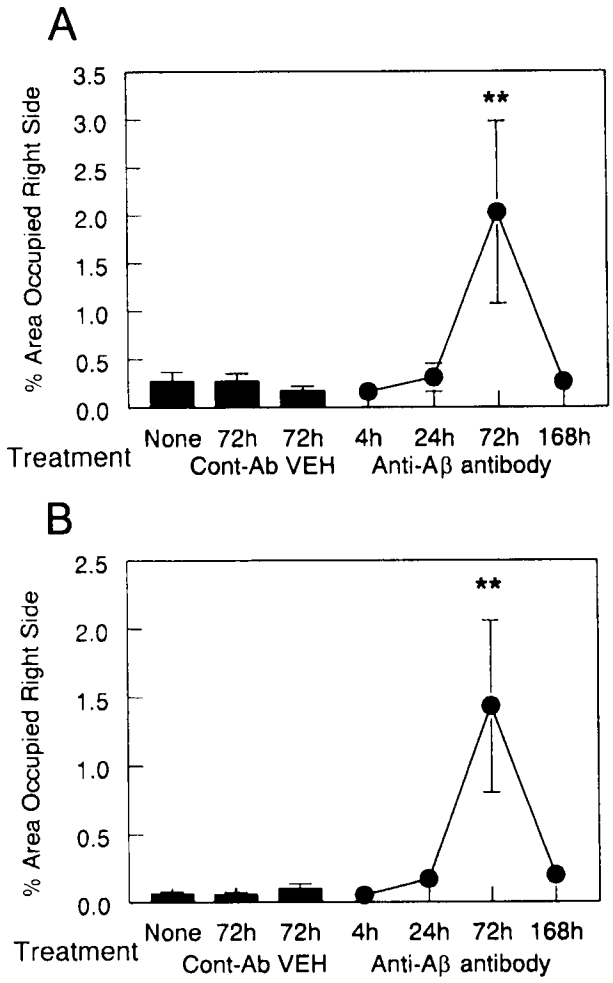

Figure 9. Anti-Aβ antibody injections result in an increase in MHC-II immunohistochemistry 3 d after injection. Data are expressed as percentage area occupied by MHC-II-positive staining in the injected hemisphere. The three bars indicate MHC-II expression in the untreated (None) group and the vehicle (VEH) and anti-HIV antibody (Cont-Ab) groups at 72 hr. The line shows the amount of MHC-II staining at 4, 24, 72, and 168 hr survival times. Increased MHC-II staining was observed in the frontal cortex (*A*) and hippocampus (*B*) at 72 hr compared with 4, 24, and 168 hr and both control groups ($^{**}p < 0.01$).

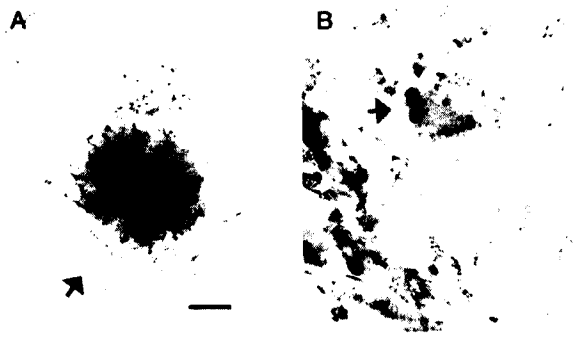

Figure 10. Anti-Aβ antibody injections result in rounded microglia in association with remaining congophilic amyloid deposits 3 d after injection. CD45 immunostaining counterstained with Congo red is shown in the hippocampus at the 72 hr time point. *A*, Typical intensely stained congophilic deposit surrounded by CD45 immunostained microglial processes, with faintly stained somata (arrow). *B*, Faintly stained congophilic deposit in the anti-Aβ antibody-injected hippocampus. Note the two rounded intensely CD45-positive cells in contact with the faintly stained deposit (*arrow*). Magnification, 600×. Scale bar, 8.33 μm.

stages of the Aβ vaccine trials resulted in a small fraction of patients developing adverse reactions consistent with inflammation of the CNS, presumably including microglial activation (Hock et al., 2002; Schenk and Yednock, 2002). Although adverse reactions to immunotherapy have been rare in the transgenic models (Pfeifer et al., 2002), it remains the best experimental system in which to understand the different components of the immune reactions to vaccines and to identify those components that cause adverse outcomes. Certainly, identification of immunotherapies that avoid the problem of deleterious CNS inflammation will be necessary if this treatment approach is to find use in the clinical setting. A better understanding of the mechanisms of antibody-mediated clearance of Aβ in the transgenic models of amyloid deposition should benefit this effort.

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of the circadian cycle in mammals. Mutational analyses of other putative clock genes will be essential for unravelling the molecular mechanisms underlying the mammalian circadian clock. These mutants will also provide useful animal models for elucidating the aetiology of and developing treatments for disorders in humans related to the sleep–wake cycle. □

Methods

Generation of *mPer2*^{Brdm1} mutant mice. We isolated a genomic clone from a mouse 129S5/SvEvBrd genomic library using a mouse *mPer2* complementary DNA probe. A targeting vector was constructed with *PGK-Neo* as the positive selection marker and *HSVtk* as the negative selection marker to delete a 2.1-kilobase (kb) fragment. We used a 6.7-kb *Bgl*II fragment as the 5′ homology region and a 4.0-kb *Kpn*I fragment as the 3′ homology region. The *HSVtk*, *PGK-Neo* and vector backbone were from pKO SelectTK, pKO SelectNeo V800 and pKO Scrambler V924 (Lexicon Genetics). Tissue culture, electroporation, mini-Southern blot analysis on embryonic stem cell colonies, generation of chimaeric and germline mice and tail DNA genotyping were done as described[27,28].

Locomotor activity monitoring and circadian phenotype analysis. Mice were housed in individual cages equipped with a running wheel in ventilated, light-tight chambers with controlled lighting. Wheel-running activity was monitored by an on-line PC using the Chronobiology Kit (Stanford Software Systems). In the LD cycle, the light was turned on at 7:00 (ZT 0) and off at 19:00 (ZT 12). The switch into constant darkness (DD) was effected by not turning on the light at the usual time. The activity records are double plotted so that each day/cycle's activity is shown both to the right and below that of the previous day/cycle. Activity is plotted in density percentile distribution (Fig. 2) or threshold (Fig. 3) format. For activity counting we used the ACTCNT program of the Chronobiology Kit. To determine the period length, an interval with a 10-day minimum during which the circadian period appeared to be stable on the activity record was analysed with a χ^2 periodogram[29] using the Stanford Chronobiology Kit. We used Fourier periodogram analysis[15] in the Chronobiology Kit to assess the strength of circadian and/or ultradian rhythmicity.

***In situ* hybridization.** Mice were killed by cervical dislocation under ambient light conditions at ZT 6 and ZT 12 and under a 15 W safety red light at ZT 18 and ZT 0/24. Specimen preparation and *in situ* hybridization with an *mPer1* and an *mPer2* probe were carried out as described[4,30]. The *mPer2* probe is outside the region deleted in the mutant. The *mPer3* probe was made from an RT–PCR product corresponding to nucleotides 480–824 (AF050182). The *Clock* probe was made from an RT–PCR product corresponding to nucleotides 1352–2080 (AF000998). Tissue was visualized by fluorescence of Hoechst dye-stained nuclei (blue in Fig. 4).

Northern blot and RT-PCR analysis. Tissues were collected and frozen in liquid nitrogen and stored at −80 °C. RNA was prepared with the RNAzol™ B RNA isolation kit (TEL-TEST). We performed northern blot analysis on total tissue RNA using denaturing formaldehyde gel. For RT–PCR analysis, first strand cDNA was generated using Moloney reverse transcriptase (BRL-GIBCO) and oligo dT-priming from total liver RNA. An aliquot of the first strand cDNA was then amplified by PCR across the deletion region with the 5′ primer CTA CCT GGT CAA GGT GCA AGA G and the 3′ primer GGT TTG AAT CTT GCC ACT GG. The RT–PCR products were then sequenced with an internal primer AGG GTA CAC TCG GGC TAT GA.

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Correspondence and requests for materials should be addressed to C.C.L. (e-mail: ching@bcm.tmc.edu)

Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse

Dale Schenk, Robin Barbour, Whitney Dunn, Grace Gordon, Henry Grajeda, Teresa Guido, Kang Hu, Jiping Huang, Kelly Johnson-Wood, Karen Khan, Dora Kholodenko, Mike Lee, Zhenmei Liao, Ivan Lieberburg, Ruth Motter, Linda Mutter, Ferdie Soriano, George Shopp, Nicki Vasquez, Christopher Vandevert, Shannan Walker, Mark Wogulis, Ted Yednock, Dora Games & Peter Seubert

Elan Pharmaceuticals, 800 Gateway Boulevard, South San Francisco, California 94080, USA

Amyloid-β peptide (Aβ) seems to have a central role in the neuropathology of Alzheimer's disease (AD)[1]. Familial forms of the disease have been linked to mutations in the amyloid precursor protein (APP) and the presenilin genes[2,3]. Disease-linked mutations in these genes result in increased production of the

42-amino-acid form of the peptide ($A\beta_{42}$)[4–8], which is the predominant form found in the amyloid plaques of Alzheimer's disease[9,10]. The PDAPP transgenic mouse, which overexpresses mutant human APP (in which the amino acid at position 717 is phenylalanine instead of the normal valine), progressively develops many of the neuropathological hallmarks of Alzheimer's disease in an age- and brain-region-dependent manner[11,12]. In the present study, transgenic animals were immunized with $A\beta_{42}$, either before the onset of AD-type neuropathologies (at 6 weeks of age) or at an older age (11 months), when amyloid-β deposition and several of the subsequent neuropathological changes were well established. We report that immunization of the young animals essentially prevented the development of β-amyloid-plaque formation, neuritic dystrophy and astrogliosis. Treatment of the older animals also markedly reduced the extent and progression of these AD-like neuropathologies. Our results raise the possibility that immunization with amyloid-β may be effective in preventing and treating Alzheimer's disease.

Our initial experiments investigated the effects of immunization against amyloid-plaque-related proteins on the development of AD-like neuropathology in young PDAPP mice, in which treatment had begun before the occurrence of significant plaque pathology (6 weeks of age). The immunogens were either synthetic human $A\beta_{42}$, the major component of β-amyloid plaques in AD and PDAPP mice, or peptides derived from the primary amino-acid sequence of serum amyloid-P component (SAP). SAP is a protein associated with amyloid plaques in AD and other amyloid diseases[13]. Two additional groups of PDAPP mice were immunized with PBS buffer or were left untreated to serve as controls. Mice received 11 immunizations over an 11-month period. We found that eight of nine PDAPP mice immunized with $A\beta_{42}$ developed and maintained serum antibody titres against $A\beta_{42}$ of greater than 1:10,000. The ninth mouse had a lower titre, of approximately 1:1,000. Cross-reactivity of the antisera was also observed against mouse amyloid-β peptide, although titres were generally an order of magnitude lower. Mice immunized with SAP peptides had antibody titres against SAP ranging from 1:1,000 to 1:10,000, with the exception of one mouse which had a titre greater than 1:10,000 (data not shown). Control animals receiving adjuvant alone had negligible titres to $A\beta_{42}$. At 13 months of age, quantitative immunohistochemical measures determined the extent of amyloid-β burden and the prevalence of neuritic plaques, astrogliosis and microgliosis.

Immunization with $A\beta_{42}$ resulted in almost complete prevention of amyloid-β deposition (Figs 1, 2). Seven of nine mice immunized with $A\beta_{42}$, including the mouse with the lowest anti-Aβ titre, had no detectable amyloid-β deposits in their brains. One mouse from this treatment group had a single isolated plaque in the six brain sections examined, whereas a second animal had a greatly reduced amyloid-β burden. Quantitative imaging of the amyloid-β burden in the hippocampus verified the near-total reduction achieved in $A\beta_{42}$-treated animals (Fig. 1). The median values of the amyloid-β burden for the PBS group (2.22%) and for the untreated control group (2.65%) were significantly greater than that of the Aβ-immunized group (0.00%, $P = 0.0005$). In contrast, the median value of amyloid-β for the group immunized with SAP peptides (5.74%), although increased, was not significantly different from that of control animals. Brain tissue from the PBS-treated control mice contained numerous amyloid-β deposits in the hippocampus (Fig. 2) and retrosplenial cortex (not shown). We observed a similar pattern of amyloid-β deposition in mice immunized with SAP peptides. Brain sections from $A\beta_{42}$-immunized mice were also stained with thioflavin S to rule out the possibility that the lack of immunohistochemically detectable plaques was due to competition by the *de novo* anti-Aβ antibodies produced by these animals (see below). Thioflavin S detected no amyloid-β deposits in these $A\beta_{42}$-immunized mice (not shown). In contrast, immunization with SAP peptides did not affect amyloid-β deposition, suggesting that

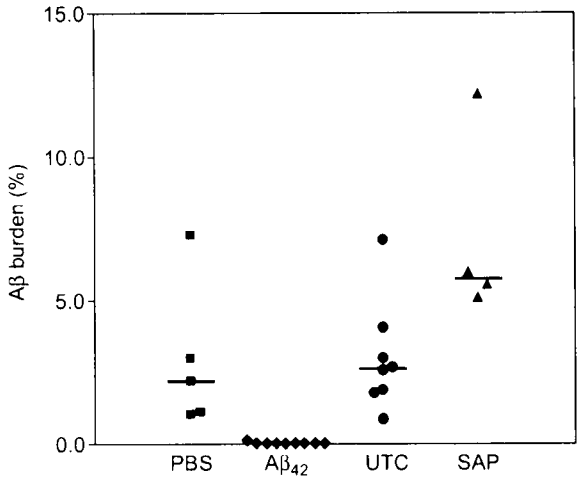

Figure 1 Reduction of Aβ burden in the hippocampus at 13 months of age in mice immunized with $A\beta_{42}$. PDAPP mice were immunized beginning at 6 weeks of age as described in Methods. The percentage of the area of the hippocampal region occupied by Aβ deposits was determined by quantitative image analysis. Values for individual mice are shown sorted by treatment group. Horizontal lines represent the median values. The $A\beta_{42}$-immunized group had significantly fewer Aβ deposits than any of the other three groups ($P = 0.001$), which are not significantly different from each other ($P > 0.05$). UTC, untreated controls; SAP, mice immunized with serum amyloid P.

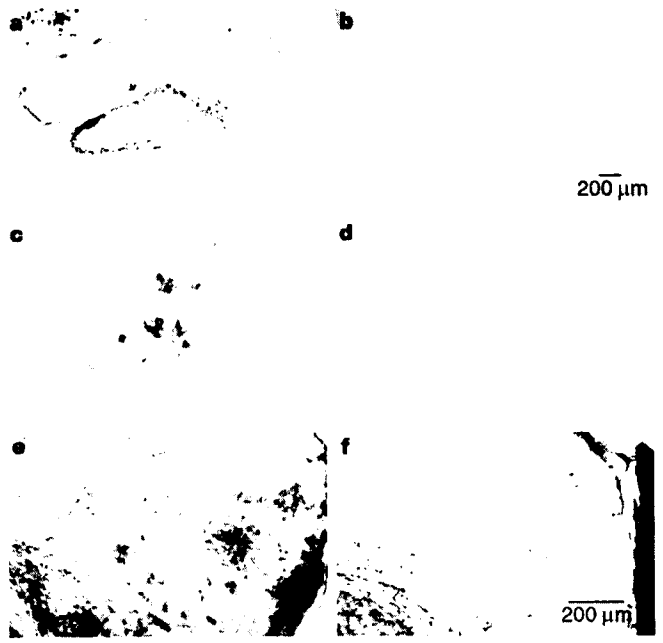

Figure 2 Hippocampal Aβ deposition, neuritic plaque formation and cortical astrocytosis in PBS- and $A\beta_{42}$-injected mice. Images from 13-month-old mice with Aβ burdens representative of the median values (see Fig. 1) of their respective groups are shown. **a**, **b**, Hippocampal Aβ plaques in PBS- (**a**) and $A\beta_{42}$-injected (**b**) mice. **a** shows abundant Aβ deposition in the outer molecular layer of the hippocampal dentate gyrus of a PBS-treated animal. **b** shows no detectable Aβ in this region of an $A\beta_{42}$-immunized mouse, a profile observed in most animals from this group. Scale bar in **b** corresponds to both **a** and **b**. **c**, **d**, Hippocampal sections from PBS- (**c**) and $A\beta_{42}$-injected (**d**) mice. Typical dystrophic neurites associated with neuritic plaques and labelled with the APP-specific monoclonal antibody 8E5 (ref. 16) were found in PBS- (**c**), but not $A\beta_{42}$-injected animals (**d**). **e**, **f**, Abundant plaque-associated astrocytosis, as determined by GFAP immunohistochemistry (Sigma), was evident in the retrosplenial cortex of PBS- (**e**) but not $A\beta_{42}$-injected (**f**) mice. Scale bar in **f** corresponds to **c**–**f**.

immune responses against plaque components *per se* are insufficient to prevent or eliminate β-amyloid plaques and neuropathology.

The brains of $A\beta_{42}$-treated mice that contained no amyloid-β deposits were also devoid of the dystrophic neurites that characterize the neuritic plaques (Fig. 2; compare c and d). Small numbers of dystrophic neurites were present in the two $A\beta_{42}$-treated mice that had detectable Aβ deposits. In contrast, all brains from SAP-injected mice and the two control groups (PBS-injected and untreated mice) had numerous neuritic plaques. Image analyses of the hippocampus demonstrated the virtual elimination of dystrophic neurites in the $A\beta_{42}$-treated mice (median, 0.00%) compared with the PBS recipients (median, 0.28%; $P = 0.0005$).

Astrocytosis, another hallmark of plaque-associated pathology in both Alzheimer's disease and PDAPP mice, was dramatically reduced in the brains of all of the $A\beta_{42}$-injected mice (Fig. 2). Brains from mice in all other groups contained numerous clusters of astrocytes that were immunoreactive to glial fibrillary acidic protein (GFAP), a finding typical of Aβ-plaque-associated gliosis (Fig. 2e).

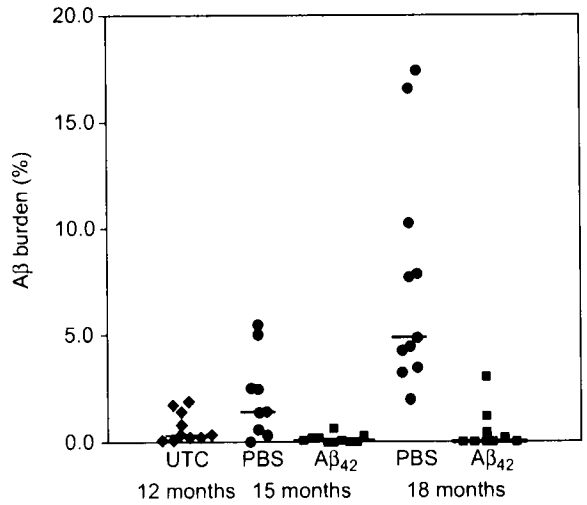

Figure 3 Quantitative image analysis of the cortical Aβ burden in older PBS- and Aβ-treated mice. Immunization of PDAPP mice was begun at 11 months of age. Amyloid burden was significantly reduced in the $A\beta_{42}$ group compared with the PBS controls at both 15 ($P = 0.003$) and 18 ($P = 0.0002$) months of age. The median value of the amyloid burden for each group is shown by the horizontal lines.

Association of amyloid plaques and reactive astrocytes was verified in a subset of GFAP-reacted sections counterstained with thioflavin S. The results of image analyses for the retrosplenial cortex verified that the reduction in astrocytosis was significant, with a median value of 1.55% for mice immunized with $A\beta_{42}$ compared with median values of greater than 6% for groups immunized with SAP peptide or PBS, or untreated control mice ($P = 0.0017$).

Sections of the mouse brains were also reacted with a monoclonal antibody specific for MAC-1 (CD11b; Chemicon), a cell-surface marker that is upregulated on activated, plaque-associated microglia. MAC-1 labelling was substantially lower in the brains of mice treated with $A\beta_{42}$ compared with the PBS control group, a finding consistent with the lack of a plaque-induced gliosis (not shown).

The almost complete absence of plaques in the brains of $A\beta_{42}$-treated mice indicates that a fundamental mechanism of amyloid plaque formation has been disrupted. Subsequent studies indicate that Aβ production itself was unaffected (data not shown). $A\beta_{42}$ immunization therefore either prevents deposition and/or enhances the clearance of Aβ from the brain. The absence of neuritic and gliotic changes suggests that the $A\beta_{42}$-immunized mice never developed the neurodegenerative lesions that typify the progression of AD-like pathology in this model. The absence of enhanced astrocytosis, in particular, suggests that the processes preventing β-amyloidosis do not in themselves cause appreciable damage to the neuropil.

The above results clearly indicate that $A\beta_{42}$ immunization essentially prevents the development of AD-like neuropathology in the PDAPP mouse. It was unclear whether $A\beta_{42}$ immunization would improve the pathological outcome if treatment were initiated when a substantial Aβ plaque burden already existed. We therefore undertook further experiments in which immunizations with $A\beta_{42}$ began at an age when many β-amyloid plaques are already present in the brains of the PDAPP mice. Immunizations were continued during a period when the extent of Aβ deposition reaches levels comparable to those of established AD[11,12]. For this study, approximately 11-month-old, heterozygotic female PDAPP mice ($n = 24$) were immunized repeatedly with $A\beta_{42}$ plus adjuvant, with an injection protocol and schedule equivalent to those used in the young PDAPP mouse study (see Methods). Similar titre responses against $A\beta_{42}$ to those seen in younger animals were generated in the older animals. As a negative control, a parallel group of 24 transgenic littermates was immunized with PBS plus adjuvant. One-half of each group was killed at 15 months of age after 4 months' treatment, and the remaining half was killed at 18 months

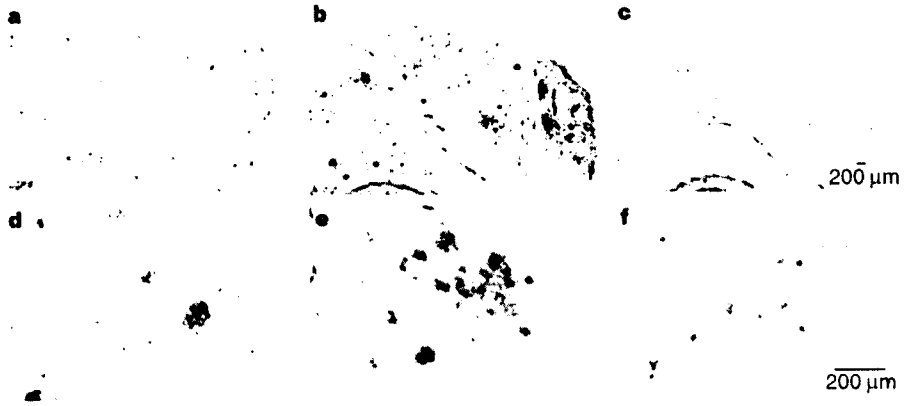

Figure 4 Reduction of cortical Aβ deposition in older PDAPP mice immunized with $A\beta_{42}$. Aβ deposits in the brains of 12-month-old untreated PDAPP mice and 18-month-old PBS- and $A\beta_{42}$-injected mice with median Aβ burdens representative of their respective treatment groups (see Fig. 3). **a**, Distribution of amyloid plaques in the frontal and retrosplenial cortices of a 12-month-old untreated PDAPP mouse, typifying the plaque burden of both PBS- and $A\beta_{42}$-injected groups at the start of the study. The plaques are shown at a high magnification in **d**. Compared with the 18-month PBS controls (**b**, **e**), Aβ deposits were significantly decreased in the 18-month $A\beta_{42}$-immunized group (**c**, **f**). Most of the cortical Aβ in brains of $A\beta_{42}$-injected mice was detected in small extracellular or cell-associated deposits (**f**) compared with the large and numerous extracellular deposits in the PBS group (**e**). Scale bar in **c** corresponds to **a**–**c**. Scale bar in **f** corresponds to **d**–**f**.

of age after 7 months' treatment. Groups of untreated PDAPP mice were also killed at ages 12, 15 and 18 months to serve as age-matched, non-immunized controls. At each time point, brains were examined by image analysis and enzyme-linked immunosorbent assay (ELISA) to determine the magnitude of the amyloid-β burden and the extent of neuritic dystrophy, astrocytosis and microgliosis.

Figure 3 shows the results of $A\beta_{42}$ treatment on cortical amyloid-β burden, determined by quantitative image analysis. The median value of cortical amyloid-β burden was 0.28% in untreated, 12-month-old PDAPP mice, a value representative of the plaque load in the experimental mice at the start of the study. At 18 months, the amyloid-β burden had increased by more than 17-fold to 4.87% in PBS-treated mice, while $A\beta_{42}$-treated mice had a greatly reduced amyloid burden of only 0.01%. The amyloid-β burden was significantly reduced in the $A\beta_{42}$ recipients at both 15 months (96% reduction; $P = 0.003$) and 18 months (>99% reduction; $P = 0.0002$). Figure 4 depicts the burden of amyloid-β in the 12-month-old PDAPP brain at the start of the experiment (Fig. 4a, d). At 18 months of age, the progression of Aβ-plaque pathology was obvious in the PBS group (Fig. 4b, e), but greatly diminished in the $A\beta_{42}$-injected mice (Fig. 4c, f). Compared with the 12-month-old untreated mice, several brains from the $A\beta_{42}$ group had fewer diffuse and mature amyloid-β deposits at 15 and 18 months, suggesting that the treatment had resulted in the clearance of pre-existing amyloid-β deposits (Fig. 4: compare a and d with c and f). Immunohistochemistry with anti-mouse immunoglobulin antibodies showed that the remaining plaques were often decorated with IgG in $A\beta_{42}$-treated but not in PBS-treated mice at both 15 and 18 months of age (data not shown).

Cortical amyloid-β deposition in PDAPP brains begins in the cingulate, frontal and retrosplenial cortices and progresses in a lateral–ventral fashion to sequentially involve the temporal and entorhinal cortices. After 3 months of treatment, amyloid-plaque pathology was diminished in the retrosplenial cortex (Fig. 5a, c) and completely absent in the entorhinal cortex (Fig. 5b, d) of the $A\beta_{42}$-injected mice. The progressive β-amyloidosis that would normally pervade the entorhinal cortex was thus halted by $A\beta_{42}$ immunization.

PDAPP mice also invariably develop heavy amyloid-β deposition in the outer molecular layer of the hippocampal dentate gyrus[11]. In a number of brains from $A\beta_{42}$-immunized mice, this pattern was considerably altered; the hippocampal deposition no longer contained diffuse amyloid-β deposits, and the banded pattern was completely disrupted (Fig. 6). Instead, unusual punctate structures were present that were reactive with anti-Aβ antibodies, and several appeared to be Aβ-containing cells (Fig. 6b). The pattern of apparent cellular labelling produced by the amyloid-β antibodies was replicated in adjacent sections by immunolabelling with antibodies directed at major histocompatibility complex (MHC) class II molecules (Fig. 6c). Phenotypically, these cells resembled activated microglia and monocytes and were occasionally found associated with the wall or lumen of blood vessels. They were not immunoreactive with antibodies that recognize T-cell (CD43, CD3e) or B-cell (CD45RA, CD45RB) surface markers (data not shown). No such cells were found in any of the PBS-treated mice.

Amyloid-β ELISA analysis of the older PDAPP mice was consistent with the immunohistochemical observations. In untreated PDAPP mice, the median level of total Aβ in the cortex at 12 months was 1,600 ng per g (wet weight); this had increased to 8,700 ng per g by 15 months[12]. At 18 months the value was 22,000 ng per g, an increase of more than 10-fold during the course of the experiment[12]. PBS-treated animals had comparable levels of total amyloid-β at 15 months (8,600 ng per g), and 19,000 ng per g at 18 months. In contrast, $A\beta_{42}$-treated animals had 81% less total amyloid-β at 15 months (1,600 ng per g) than the PBS-immunized group. Significantly less total amyloid-β (5,200 ng per g) was found at 18 months when the $A\beta_{42}$ and PBS groups were compared, representing a 72% reduction ($P = 0.0001$) in the amyloid-β that would have otherwise been present. Similar results were obtained when cortical levels of $A\beta_{42}$ were compared, namely that the $A\beta_{42}$-treated group contained much less $A\beta_{42}$, and the differences between the $A\beta_{42}$ and PBS groups were significant at 15 months ($P = 0.04$) and 18 months ($P = 0.0001$). In contrast, cortical levels of APP decreased by less than 10% of those in 12-month-old untreated animals (data not shown)[12]. These findings argue against the possibility that the reduction in amyloid-β deposition seen in the treated mice is due to an alteration in APP metabolism.

The progression of neuritic pathology was significantly reduced in the frontal cortex of $A\beta_{42}$-treated compared with PBS-treated mice (Table 1). At 15 months of age, the neuritic plaque burden in

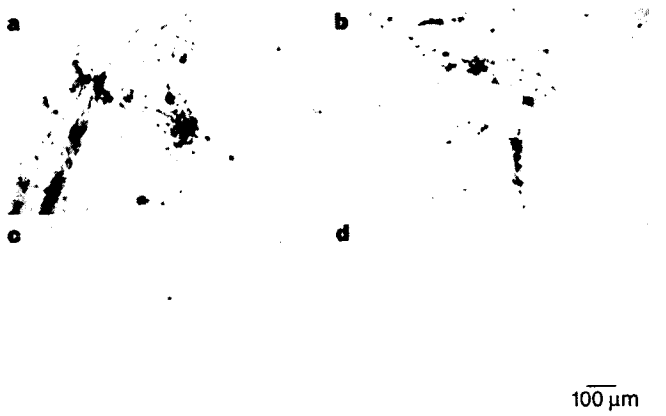

Figure 5 Reduction of Aβ burden in the entorhinal and retrosplenial cortex of older PDAPP mice following Aβ injection. Aβ deposition in the retrosplenial (RSC) and entorhinal (EC) cortices of 15-month-old PBS- (**a**, **b**) and $A\beta_{42}$- (**c**, **d**) injected mice with Aβ burdens representative of the median values of their respective groups. Aβ deposition was greatly reduced in the RSC of $A\beta_{42}$-injected mice compared with the PBS group (compare **a** and **c**). No Aβ was detected in the EC of $A\beta_{42}$-injected mice (**d**), in contrast to the PBS group (**b**). Scale bar in **d** corresponds to all panels.

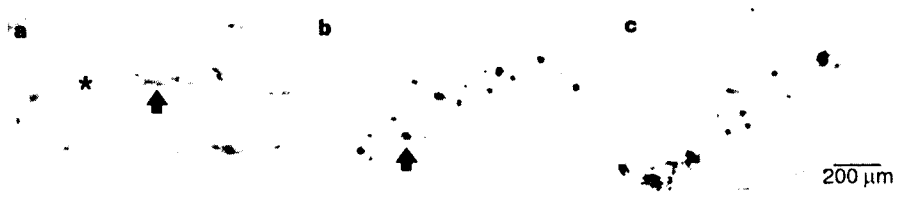

Figure 6 Altered Aβ burden in the hippocampus of older $A\beta_{42}$-treated mice. Distribution of hippocampal Aβ in $A\beta_{42}$-injected brains (**b**), compared with the PBS group (**a**) at 18 months of age. In the PBS group, the characteristic appearance of diffuse (asterisk) and compacted (arrow) Aβ deposits was evident (**a**). This pattern was markedly altered in a number of $A\beta_{42}$-immunized mice (**b**), with the absence of diffuse deposits and an unusual punctate pattern of Aβ immunoreactivity associated with cells (**b**, arrow). The distribution of MHC II-labelled (Pharmigen) cells in a near-adjacent section (**c**) corresponded to the pattern of Aβ-positive cellular labelling shown in **b**. No such obvious cell Aβ staining was found in the PBS group (compare **a** and **b**). Scale bar in **c** corresponds to all panels.

Table 1 Image analysis of neuritic plaque and astrocytic burden in $A\beta_{42}$-treated PDAPP mice

	15 months		18 months	
	PBS	$A\beta_{42}$-treated	PBS	$A\beta_{42}$-treated
Neuritic plaque (%)	0.32	0.02	0.49	0.22
Astrocytosis (%)	4.26	1.89	5.21	3.20

Quantitative image analysis of neuritic plaques and astrocytosis was performed using antibody 8E5 to human APP and anti-GFAP, respectively. Methods are described in Fig. 1 legend. Reduction of neuritic plaque burden at ages 15 and 18 months by $A\beta_{42}$ treatment was statistically significant ($P = 0.03$ and 0.01, respectively), as was reduction of astrocytosis at the same time points ($P = 0.01$ and 0.03, respectively).

the $A\beta_{42}$-treated mice was reduced by 84% compared with the PBS group (0.05% and 0.32%; $P = 0.03$). Reduction in the neuritic-plaque pathology was similarly maintained between the two groups at 18 months of age, where the degree of neuritic dystrophy was reduced by 55% in the $A\beta_{42}$-treated mice (0.22% and 0.49%; $P = 0.01$).

Reactive astrocytosis was also significantly reduced in the retrosplenial cortex of $A\beta_{42}$-treated mice compared with PBS-treated mice at both 15 and 18 months of age. The per cent of astrocytosis in the PBS group increased between 15 and 18 months from 4.29% to 5.21%. $A\beta_{42}$ treatment suppressed the development of astrocytosis at both time points to 1.89% and 3.2%, respectively. These differences represent a 56% reduction ($P = 0.01$) at 15 months of age and a 34% decrease ($P = 0.03$) at 18 months of age in the $A\beta_{42}$-treated group.

In summary, immunization with $A\beta_{42}$ greatly reduced the development of the AD-like pathology that otherwise occurs in the PDAPP mouse. Immunization preceding plaque development profoundly affected the occurrence of new lesions, as the progression of β-amyloidosis and associated neuropathology was essentially wholly blocked, as seen both in the entire brain of the young animals and in at-risk brain regions of the older animals. Amyloid-β immunization also significantly retarded the progression of existing pathology in affected regions of the older animals. Outcomes of Aβ-plaque burden, neuritic dystrophy and gliosis were all significantly improved by $A\beta_{42}$ treatment in both young and old animals. In addition, the mechanism resulting in plaque reduction did not seem to produce any obvious signs of damage to the neuropil of $A\beta_{42}$-immunized animals. Histological examination of several organs, including brain and kidney, revealed no signs of immune-mediated complications, despite the high levels of human APP expressed in these tissues and the significant antibody titre to endogenous mouse Aβ peptide (data not shown).

To our knowledge, this is the first report of a clinically relevant treatment that reduces the progression of AD-like neuropathology in a transgenic animal model of the disease. Although it remains unproven, it is not unreasonable to expect that a similar reduction of neuropathology in AD patients would be of clinical benefit. Although our understanding of the precise aspects of the immune response that result in reduced pathology is incomplete, we have shown that $A\beta_{42}$ immunization results in the generation of anti-Aβ antibodies and that Aβ-immunoreactive monocytic/microglial cells appear in the regions of remaining plaques. Thus, one possible mechanism of action is that anti-Aβ antibodies facilitate clearance of amyloid-β either before deposition, or after plaque formation, by triggering monocytic/microglial cells to clear amyloid-β using signals mediated by F_c receptors.

It has been suggested that a chronic inflammatory state exists in the brain of patients with Alzheimer's disease: specifically the levels of complement, cytokines and acute-phase proteins are raised[14]. These observations have led to the hypothesis that anti-inflammatory regimens might be of therapeutic value. The findings presented here argue that an alternative approach, one that augments a highly specific immune response, can markedly reduce pathology in an animal model of the disease. Collectively, the results suggest that amyloid-β immunization may prove beneficial for both the treatment and prevention of Alzheimer's disease. □

Methods

Immunization procedures. Aβ peptide was freshly prepared from lyophilized powder for each set of injections. For immunizations, 2 mg $A\beta_{42}$ (human $A\beta_{1-42}$; US Peptides) was added to 0.9 ml deionized water and the mixture was vortexed to generate a relatively uniform suspension. A 100-μl aliquot of 10 × PBS (where 1 × PBS is 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was added. The suspension was vortexed again and incubated overnight at 37 °C for use the next day. Serum amyloid-P component immunogens were prepared using mouse SAP amino acids 77–85 and 164–173, each conjugated to sheep anti-mouse IgG (Jackson Immunochemicals) as described[15]. $A\beta_{42}$ or SAP peptides (100 μg antigen per injection) were emulsified 1:1 (v/v) with complete Freund's adjuvant for the first immunization, followed by a boost in incomplete Freund's adjuvant at 2 weeks, and monthly thereafter. $A\beta_{42}$ or SAP in PBS alone was injected from the fifth immunization onward. Titres were determined by serial dilutions of sera against either aggregated $A\beta_{42}$ or SAP protein which had been coated onto microtitre wells. Detection used goat anti-mouse immunoglobulin conjugated to horseradish peroxidase and slow-TMB (3,3″,5,5′-tetramethyl benzidine; Pierce) substrate. Titres were defined as the dilution yielding 50% of the maximal signal.

Neuropathology quantification. To quantify amyloid burden, PDAPP mouse brain tissue was fixed in 4% paraformaldehyde, cut to 40-μm coronal sections and reacted with an anti-Aβ biotinylated antibody, 3D6, as described previously[11]. Quantitative image analysis was performed using a Videometric 150 Image Analysis System (Oncor) linked to a Nikon Microphot-FX microscope through a CCD video camera. The image of the immunoreacted section was stored in a video buffer and a specific brain region (hippocampus or cortex) was manually outlined and the total pixel area occupied by the structure determined. A monochromatic-based threshold was set to select pixels corresponding to immunolabelled structures. The per cent of the brain region occupied by the labelled pixels was then calculated. For all image analyses, six sections at the level of the dorsal hippocampus, each separated by consecutive 240-μm intervals, were evaluated for each animal. In all cases, the treatment status of the animals was unknown to the observer. Mann–Whitney nonparametric analysis was performed using Statview software (SAS Institute, Cary, NC). Similar methodologies were used to quantify neuritic dystrophy and gliosis. Specific reagents are indicated in the appropriate figures.

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Correspondence and requests for materials should be addressed to D.S. (e-mail: dschenk@elanpharma.com).

Peripherally administered antibodies against amyloid β-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease

FRÉDÉRIQUE BARD, CATHERINE CANNON, ROBIN BARBOUR, RAE-LYN BURKE, DORA GAMES, HENRY GRAJEDA, TERESA GUIDO, KANG HU, JIPING HUANG, KELLY JOHNSON-WOOD, KAREN KHAN, DORA KHOLODENKO, MIKE LEE, IVAN LIEBERBURG, RUTH MOTTER, MINH NGUYEN, FERDIE SORIANO, NICKI VASQUEZ, KIM WEISS, BRENT WELCH, PETER SEUBERT, DALE SCHENK & TED YEDNOCK

Elan Pharmaceuticals, 800 Gateway Boulevard, South San Francisco, California 94080, USA
Correspondence should be addressed to F.B.; email: fbard@elanpharma.com

One hallmark of Alzheimer disease is the accumulation of amyloid β-peptide in the brain and its deposition as plaques. Mice transgenic for an amyloid β precursor protein (APP) mini-gene driven by a platelet-derived (PD) growth factor promoter (PDAPP mice), which overexpress one of the disease-linked mutant forms of the human amyloid precursor protein, show many of the pathological features of Alzheimer disease, including extensive deposition of extracellular amyloid plaques, astrocytosis and neuritic dystrophy[1,2]. Active immunization of PDAPP mice with human amyloid β-peptide reduces plaque burden and its associated pathologies[3]. Several hypotheses have been proposed regarding the mechanism of this response[4,5]. Here we report that peripheral administration of antibodies against amyloid β-peptide, was sufficient to reduce amyloid burden. Despite their relatively modest serum levels, the passively administered antibodies were able to enter the central nervous system, decorate plaques and induce clearance of preexisting amyloid. When examined in an *ex vivo* assay with sections of PDAPP or Alzheimer disease brain tissue, antibodies against amyloid β-peptide triggered microglial cells to clear plaques through Fc receptor-mediated phagocytosis and subsequent peptide degradation. These results indicate that antibodies can cross the blood–brain barrier to act directly in the central nervous system and should be considered as a therapeutic approach for the treatment of Alzheimer disease and other neurological disorders.

We administered antibodies against amyloid β-peptide (Aβ) by intraperitoneal injection to mice transgenic for an Aβ precursor protein (APP) mini-gene driven by a platelet-derived (PD) growth factor promoter (PDAPP mice). In the first experiment, 8- to 10-month-old heterozygous PDAPP mice ($n = 8$) received either PBS, one of two different mouse monoclonal antibodies against Aβ (10D5 or 21F12) or a polyclonal immunoglobulin (Ig) fraction obtained from mice immunized with the 42-amino-acid form of Aβ (pabAβ_{1-42}). The mice received weekly injections of antibody for six months, maintaining a constant antibody serum concentration throughout the course of the experiment. We used quantitative image analysis to determine amyloid burden and enzyme-linked immunoassay (ELISA) to determine Aβ_{42} levels in the cortex, as described[3]. Relative to control-treated mice, the polyclonal immunoglobulin fraction against Aβ and one of the monoclonal antibodies (10D5) reduced plaque burden by 93% and 81%, respectively (Fig. 1*a*, $P < 0.005$). There were similar reductions in cortical levels of Aβ_{42} by ELISA measurements (6200, 4890 and 13800 ng/g tissue for 10D5, pabAβ_{1-42} and PBS, respectively). Although 21F12 seemed to have a relatively modest effect on plaque burden, there was no substantial reduction as determined by ELISA measurements (13,580 ng/g). The effect of pabAβ_{1-42} on plaque burden was demonstrated in micrographs of brain sections obtained from mice having the median level of plaque burden within their respective groups. Most of the diffuse deposits and many of the larger compacted plaques were absent in mice treated with pabAβ_{1-42} compared to those in the control groups.

In a second study, we repeated 10D5 treatment and tested two additional antibodies against Aβ, 3D6 and 16C11 (Fig. 1*c*). Control groups received either PBS or an irrelevant isotype-matched antibody (TM2a). The experimental design was the same as in the previous study except for the age of the mice (11.5–12 months old). Once again, after 6 months of treatment, 10D5 reduced plaque burden by greater than 80% compared with that of either the PBS or isotype-matched antibody controls ($P = 0.003$). Treatment with 3D6 was equally effective, producing an 86% reduction in plaque burden ($P = 0.003$). In contrast, 16C11 failed to have any effect on plaque burden. Unlike actively immunized mice, mice receiving exogenous antibodies against Aβ did not demonstrate a T-cell proliferative response to Aβ when their splenocytes were examined *in vitro*, indicating that a T-cell response is not required for amyloid plaque clearance (data not shown). These results indicate that in the absence of T-cell immunity, antibodies against Aβ peptide are sufficient to decrease amyloid deposition in PDAPP mice.

To determine whether the peripherally administered antibodies against Aβ had entered the central nervous system (CNS) to act directly on plaques, we examined brain sections taken from saline-perfused mice at the end of the second study. We exposed unfixed cryostat brain sections to a fluorochrome-tagged reagent against mouse immunoglobulin. Plaques within the brains of the 10D5- and 3D6-treated groups were strongly 'decorated' with antibody, whereas there was no staining in the 16C11-treated group (Fig. 2*a*, top). To show the full extent of plaque deposition, we immunostained serial sections of each brain with an exogenous antibody against Aβ, followed by the secondary reagent (Fig. 2*a*, bottom). After peripheral administration, 10D5 and 3D6 had gained access to most plaques within the CNS where they

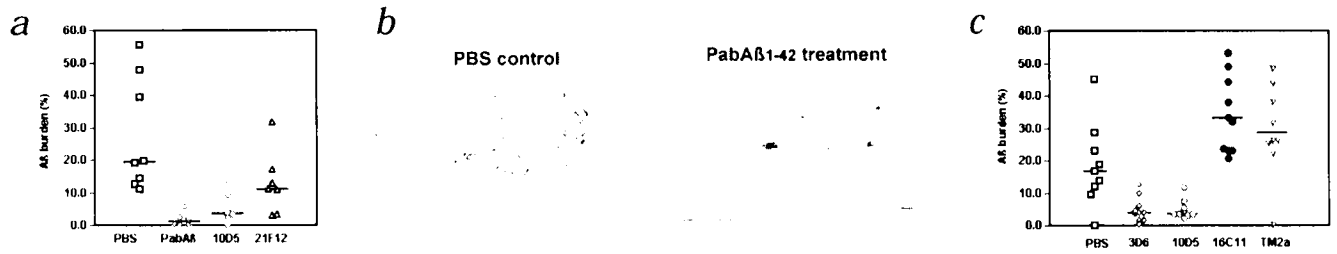

Fig. 1 Aβ burden in the frontal cortex of PDAPP mice is reduced after 6 months of treatment with antibodies against Aβ. ***a*** and ***c***, Percentage of frontal cortex area occupied by Aβ deposits shown in individual mice sorted by treatment group (n = 8). Horizontal lines indicate median values. ***b***, Brain sections of the cortex and hippocampus obtained from mice with the median level of plaque burden in their respective groups. Scale bar represents 250 μm.

may have directly triggered amyloid clearance. It is likely that 16C11 also had access to the plaques but was unable to bind (described below).

We undertook a separate study to determine if antibody treatment resulted in the clearance of preexisting amyloid or simply prevented formation of new plaques. We administered 3D6 or control antibody to 13-month-old heterozygous PDAPP mice, then examined the brains for total plaque burden after 3 and 35 days of treatment. Although there was no obvious change in the number of large plaques over the short treatment period (Fig. 2*b*), there seemed to be a reduction in diffuse amyloid and small aggregates of Aβ (Fig. 2*b*, inset). By image analysis of the frontal cortex, nearly 60% of the small plaques and diffuse amyloid, corresponding to pixels with intermediate to low intensity, had been eliminated during the intervening 32 days of treatment (Fig. 2*c*; P = 0.001). These results confirm that the antibody treatment triggered clearance of preexisting amyloid.

To further examine the effect of antibodies on plaque clearance, we established an *ex vivo* assay in which primary microglial cells were cultured with unfixed cryostat sections of either PDAPP mouse or human Alzheimer disease (AD) brains. After 24 hours, we fixed, permeabilized and immunostained the cultures to follow the fate of amyloid β-peptide, and visualized the exogenous microglial cells with a nuclear stain. In PDAPP brain sections assayed in the presence of 16C11 (one of the antibodies against Aβ that was not efficacious *in vivo*), amyloid β-protein plaques remained intact and there was no phagocytosis. In contrast, after culture of adjacent sections in the presence of 10D5, amyloid deposits were mostly eliminated and the microglial cells showed many phagocytic vesicles containing Aβ (Fig. 3*a*, top). We obtained identical results with AD brain sections: 10D5 induced phagocytosis of AD plaques, whereas 16C11 was inactive (Fig. 3*a*, bottom). Furthermore, the assay was equally effective with either mouse or human microglial cells, and with mouse, rabbit or primate antibodies against Aβ (data not shown).

By comparison of six different antibodies tested in both systems, the *ex vivo* assay was predictive of *in vivo* efficacy (Table 1). Antibodies 10D5, 3D6 and pabAβ_{1-42} were all active in the *ex vivo* assay and demonstrated efficacy *in vivo*. In contrast, 16C11, 21F12 and the control antibody TM2a were inactive in

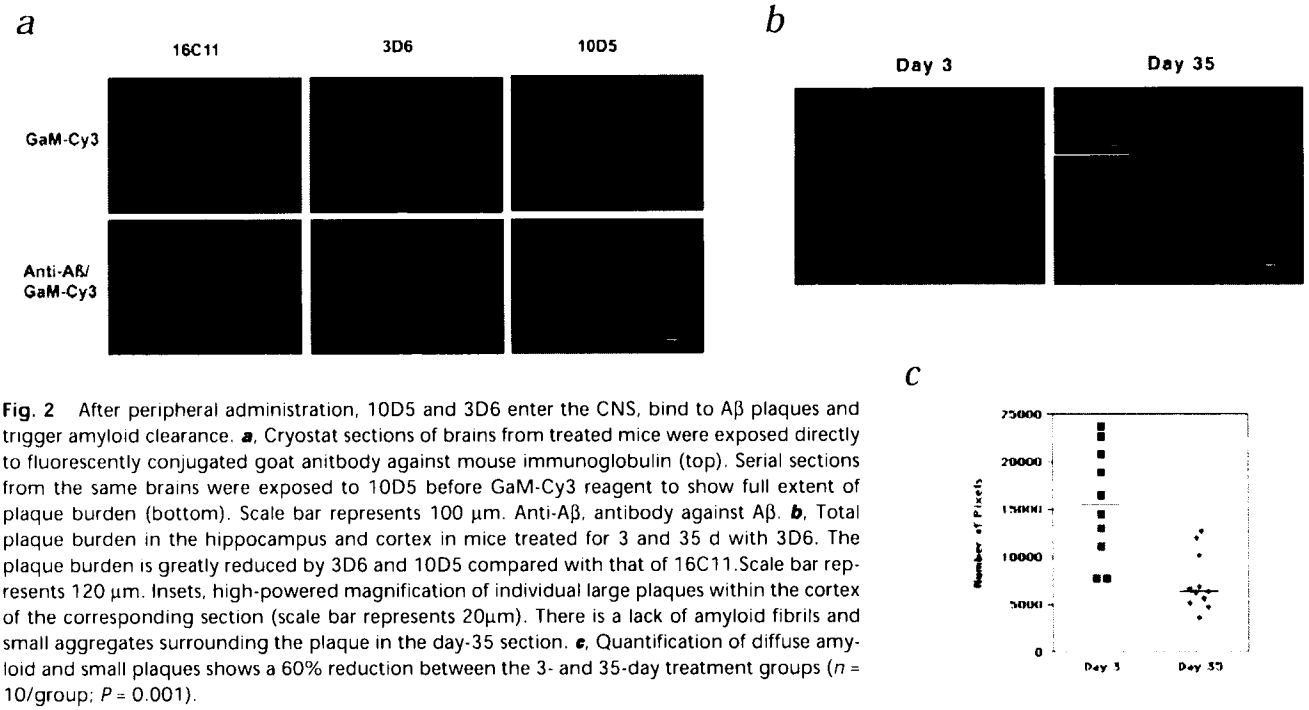

Fig. 2 After peripheral administration, 10D5 and 3D6 enter the CNS, bind to Aβ plaques and trigger amyloid clearance. ***a***, Cryostat sections of brains from treated mice were exposed directly to fluorescently conjugated goat anitbody against mouse immunoglobulin (top). Serial sections from the same brains were exposed to 10D5 before GaM-Cy3 reagent to show full extent of plaque burden (bottom). Scale bar represents 100 μm. Anti-Aβ, antibody against Aβ. ***b***, Total plaque burden in the hippocampus and cortex in mice treated for 3 and 35 d with 3D6. The plaque burden is greatly reduced by 3D6 and 10D5 compared with that of 16C11.Scale bar represents 120 μm. Insets, high-powered magnification of individual large plaques within the cortex of the corresponding section (scale bar represents 20μm). There is a lack of amyloid fibrils and small aggregates surrounding the plaque in the day-35 section. ***c***, Quantification of diffuse amyloid and small plaques shows a 60% reduction between the 3- and 35-day treatment groups (n = 10/group; P = 0.001).

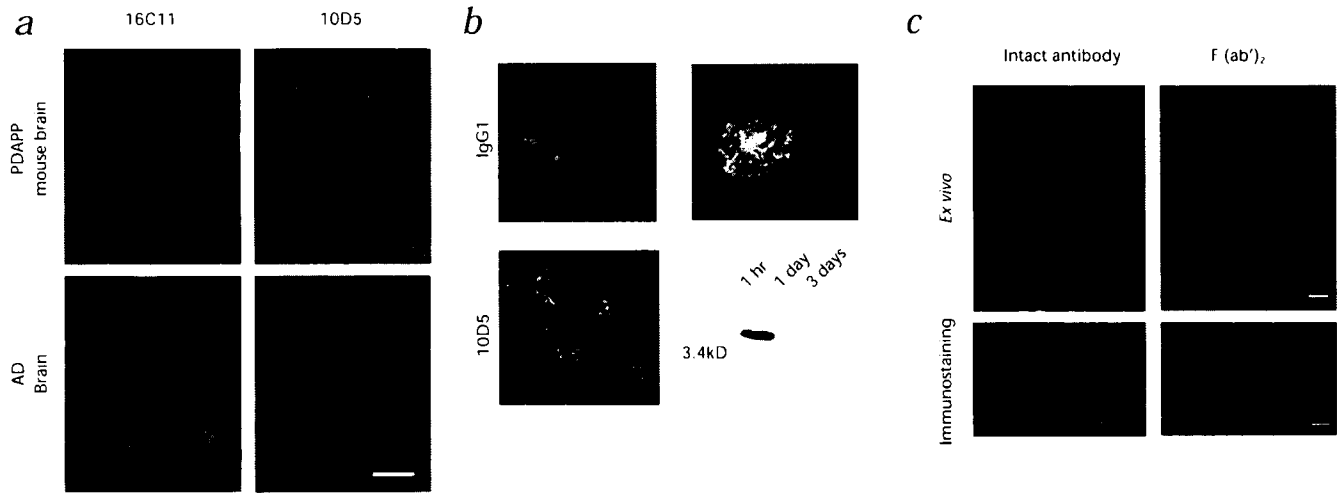

Fig. 3 Fc receptor-mediated phagocytosis of Aβ in an *ex vivo* assay. ***a***, Mouse microglia cultured with unfixed cryostat sections of PDAPP (top) or AD (bottom) brain in the presence of antibodies against Aβ. In the presence of 10D5, Aβ (red) localizes to vesicles within the membrane boundaries of microglial cells; in the presence of 16C11, plaques remain intact and there is no evidence of cellular staining. Scale bar represents 10 μm. ***b***, Confocal microscopy shows that in the presence of control IgG1 (top), microglial cells (red) are in a confocal plane above the tissue section and Aβ (green) remain in plaques within the tissue plane (different planes of the same field are shown here). In the presence of 10D5 (bottom left), nearly all Aβ is localized within the microglial cells. Bottom right, western blot analysis with a polyclonal antibody against Aβ shows complete degradation of Aβ within 3 d (left margin, molecular size marker). ***c***, Fc receptor-mediated phagocytosis of Aβ, shown by *ex vivo* assay in the presence of intact or $F(ab')_2$ fragments of 3D6 (top). Scale bar represents 50 μm. Aβ plaques in consecutive sections are equally 'decorated' with intact 3D6 and its $F(ab')_2$ fragments (bottom). Scale bar represents 300 μm.

both assays. Comparison of other antibody characteristics showed that whereas 16C11 and 21F12 bound to aggregated synthetic Aβ peptide with high avidity, they were unable to bind to plaques in unfixed brain sections. This result is consistent with the inability of these two antibodies to decorate plaques after *in vivo* administration (as shown for 16C11 in Fig. 2*a*, top) and explains their inability to trigger plaque clearance. We found no correlation between antibody efficacy and affinity for soluble Aβ: 3D6 and the inactive antibodies captured soluble Aβ with moderate to high affinity, whereas 10D5 could not capture the soluble peptide. These results indicate that recognition and clearance of deposited Aβ is an important component of antibody efficacy *in vivo* and may be more important than a mechanism involving the prevention of plaque deposition by recognition of soluble Aβ.

We then used confocal microscopy to confirm that Aβ was internalized during the course of the *ex vivo* assay (Fig. 3*b*). In the presence of control antibody, the exogenous microglial cells (red) remained in a confocal plane above the tissue section and contained no phagocytic vesicles, whereas Aβ (green) remained in plaques within the tissue plane (Fig. 3*b*, top). In the presence of 10D5, nearly all plaque amyloid was contained in vesicles within the exogenous microglial cells (Fig. 3*b*, bottom). This result is consistent with the earlier finding of intracellular Aβ immunoreactivity within macrophage and microglial cells in the CNS of immunized mice[3]. To determine the fate of the internalized peptide, we assessed 10D5-treated cultures by western blot analysis (Fig. 3*b*, bottom right). At 1 hour, when no phagocytosis had yet occurred, reaction with a polyclonal antibody against Aβ showed a strong 4-kDa band, corresponding to Aβ peptide. Aβ immunoreactivity decreased at day 1 and was absent by day 3. There was no degradation of Aβ staining in cultures with control IgG1. Thus, in contrast to what has been reported for Aβ with other microglial scavenging pathways[6,7], antibody-mediated phagocytosis of Aβ led to its degradation.

To determine if phagocytosis in the *ex vivo* assay was mediated by Fc, we prepared $F(ab')_2$ fragments of 3D6. Although the antibody fragments retained their full ability to react with plaques (Fig. 3*c*, bottom), they were unable to trigger microglial cell phagocytosis (Fig. 3*c*, top). In addition, phagocytosis induced by the whole 3D6 antibody was inhibited by antibodies specific for Fc receptors on either human or mouse microglial cells (data not shown). These results indicate that *in vivo* clearance of Aβ occurred through Fc receptor-mediated phagocytosis.

In summary, we have shown that passively administered antibodies against Aβ peptide reduced the extent of plaque deposition in a mouse model of Alzheimer dis-

Table 1 The *ex vivo* assay as predictor of *in vivo* efficacy.

Antibody	Isotype	Avidity for aggregated Aβ (pM)	Affinity for soluble Aβ (nM)	Binding to Aβ plaques	*Ex vivo* efficacy	*In vivo* efficacy
monoclonal						
3D6	IgG2b	470	< 30	+	+	+
10D5	IgG1	43	no capture	+	+	+
16C11	IgG1	90	110	-	-	-
21F12	IgG2a	500	80	-	-	-
TM2a	IgG1	-	-	-	-	-
$PabA\beta_{1-42}$	mix	600	nd	+	+	+

The affinity of 10D5 was too low to capture soluble Aβ and could not be measured in this assay. nd, not done.

ease. Antibody entry into the CNS was not due to abnormal leakage of the blood–brain barrier, as there was no increase in vascular permeability in PDAPP mice. In addition, the concentration of endogenous immunoglobulins in the brain parenchyma of aged PDAPP mice was the same as in nontransgenic mice, representing 0.1% of the antibody concentration in serum (regardless of isotype; data not shown). Similar findings have been published for antibody levels in human cerebrospinal fluid[8]. These studies indicate that monoclonal antibodies are able to enter the CNS at therapeutically relevant levels, and that antibodies may be considered not only for the treatment of Alzheimer disease, but possibly for other CNS disorders as well.

Methods

Immunization procedures. Monoclonal antibodies against Aβ were raised against synthetic peptide fragments derived from different regions of Aβ, and coupled to a carrier protein as described[9,10]. Polyclonal immune sera were pooled from 100 mice that had been immunized with full length $Aβ_{1-42}$, and the immunoglobulin fraction was isolated by standard ammonium sulfate precipitation. After purification, all antibodies were dialyzed against PBS and had a final endotoxin level of < 1 endotoxin unit (EU), as measured by the limulus amoebocyte gel clot assay (Associates of Cape Cod, Cape Cod, Massachusetts). Antibody titers in serum were determined weekly as described[3].

***Ex vivo* assay.** Cryostat sections (10 μm in thickness) of PDAPP mouse or human AD brains (postmortem interval, less than 3 h) were 'thaw-mounted' onto polylysine-coated, round glass coverslips and placed in wells of 24-well tissue culture plates. The coverslips were washed twice with assay medium consisting of hybridoma-serum free medium (H-SFM; Life Technologies) plus 1% FBS, glutamine, penicillin/streptomycin, and 5 ng/ml recombinant mouse GM-CSF granulocyte–monocyte colony-stimulating factor)(R&D Systems, Minneapolis, Minnesota). Antibodies (control or against Aβ) were added at a 2X concentration (5 μg/ml final) for 1 h. Microglial cells were then seeded at a density of 0.8×10^6 cells/ml assay medium. In some experiments, medium contained 10 μg/ml soybean trypsin inhibitor (Life Technologies). The cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 for 24 h or longer. After incubation, cultures were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X100. Sections were stained with biotinylated 3D6, followed by streptavidin/Cy3 conjugate (Jackson ImmunoResearch, West Grove, Pennsylvania). Cultures were observed with an inverted fluorescent microscope (TE300; Nikon, Melville, New York) and photomicrographs were taken with a SPOT digital camera using SPOT software (Diagnostic Instruments, Sterling Heights, Michigan). For western blot analysis, cultures were extracted with 8 M urea, diluted 1:1 in reducing tricine sample buffer, and loaded onto a 16% tricine gel (Novex, San Diego, California). After transfer onto Immobilon, blots were exposed to 5 μg/ml $pabAβ_{42}$, followed by horseradish peroxidase-conjugated antibody against mouse, and were developed with an ECL kit (Amersham).

Microglia culture. Microglial cells were obtained from cerebral cortices of neonate DBA/2N mice (1–3 days old). Cortices were mechanically dissociated in Hanks' balanced salt solution with 50 μg/ml DNase I (both from Sigma). Dissociated cells were filtered through a 100 μm cell strainer (Falcon, Heidelberg, Germany) and centrifuged at 200*g* for 5 min. Pellets were resuspended in growth medium (high-glucose DMEM, 10% FBS and 25 ng/ml recombinant mouse granulocyte–monocyte colony-stimulating factor), and cells were plated at a density of two brains per T-75 plastic culture flask. After 7–9 d, the flasks were rotated at 200 rpm using a Lab-Line orbital shaker with a 19-mm orbit for 2 h at 37 °C. Cell suspensions were centrifuged at 200*g* and resuspended in assay medium.

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